EVALUATION OF BONE BIOCHEMICAL MARKERS AND INFLAMMATORY MARKERS IN YEARLINGS FED VARYING RATIOS OF OMEGA-6 AND OMEGA-3 POLYUNSATURATED FATTY ACIDS

A Thesis

by

TRINETTE NOEL ROSS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2006

Major Subject: Animal Science

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Approved by:

Co-Chairs of Committee,	Gary Potter
	Pete Gibbs
Committee Members,	Brett Scott
	Wayne Sampson
Head of Department,	Gary Acuff

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ABSTRACT

Evaluation of Bone Biochemical Markers and Inflammatory Markers in Yearlings Fed Varying Ratios of Omega-6 and Omega-3 Polyunsaturated Fatty Acids.

(December 2006)

Trinette Noel Ross, B.S., Montana State University

Co-Chairs of Advisory Committee: Dr. Gary Potter
Dr. Pete Gibbs

Diets formulated to contain varying ratios of omega 6 to omega 3 fatty acids were fed to exercising yearlings to evaluate bone activity and inflammatory response. Nine Quarter Horse yearlings were arranged within a triplicated 3 X 3 Latin Square experimental design and fed one of three diets. Exercise protocol was designed to stimulate sub-clinical inflammation and normal bone response.

Body weight and physical growth measurements were not different between groups ($P > 0.05$), and feed intake was similar between groups ($P > 0.05$).

Horses consuming soybean oil (SBO) diet had lower fatty acid profiles (% by weight) of C16:0 and C16:1 ($P < 0.05$) when compared to horses consuming either corn oil (CO) or menhaden/corn oil (MCO) diets. Though numerically different, percentage changes in C16:0 and C16:1 were not different between diets ($P < 0.05$). Horses consuming MCO had significantly higher measurements of C20:4, C20:5 and C22:6 over the 28 day period when compared to horses consuming SBO or CO. Percent change in mean concentrations of C20:5 were significantly different between the MCO group and

the SBO group ($P < 0.05$) with no observed difference between MCO and CO treatment groups.

Overall mean carboxyterminal telopeptide of type I collagen (ICTP) concentrations did not differ between diets ($P > 0.05$) nor was there a significant change from baseline values when compared to day 28 of the period.

Mean Osteocalcin (OC) concentrations did not differ between treatments ($P > 0.05$). Numerically, OC levels were lower after 14 days, with subsequent increases occurring from day 14 to day 28; however, there was no significant day effect ($P > 0.05$).

Mean measurements of PGE_2 and fibrinogen, the two inflammation markers evaluated, did not differ among groups ($P > 0.05$). However, when fibrinogen data were normalized, horses consuming SBO had a significantly lower change in baseline values of fibrinogen compared to horses fed CO or MCO diets ($P< 0.05$).

In general, horses fed SBO exhibited reduced levels of the inflammatory marker fibrinogen ($P< 0.05$). No other variable evaluated was influenced by the supplementation of varying ratios of polyunsaturated fatty acids into the equine diet.

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CHAPTER I

INTRODUCTION

The evaluation of omega 6 and omega 3 polyunsaturated fatty acids (PUFA) and their application in the mammalian diet has been the subject of nutritional inquires for years. Research has hypothesized that elevated omega 3 fatty acids in the diet may provide a protective effect to inflammatory responses in humans (Kettler, 2001) as well as reducing the incidence of bone loss in geriatric subjects (Terano, 2001).

The evaluation of polyunsaturated fatty acids in the equine diet is limited; however, its popularity has increased in recent years with the development of more palatable fat sources. In the limited information available on essential fatty acid in the equine diet; it has been established that elevating omega 3 PUFA content of the diet results in a dose-dependent incorporation of those fatty acids into equine cellular membranes and bloodstream (King et al., 2005). Most research evaluating omega 3 PUFA mediation of inflammatory processes in horses have relied on in vitro methodology. Very few queries have used in vivo procedures to assess PUFA response to biological processes in the horse. What information that is available appears to be promising. Wilson et al. (2003) determined that exercising horses fed soybean oil expressed lower fibrinogen concentrations compared to the fibrinogen concentrations of exercising horses fed corn oil.

Supplementation of omega 3 fatty acids have been confirmed to significantly influence constituents of rat bone; increasing formation markers such as bone alkaline

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phosphatase (Watkins et al. 2000), decreasing bone PGE_2 concentrations (Green et al., 2004) and increasing calcium concentrations in the bone (Claassen et al., 1995).

At present, there has been minimal research investigating the supplementation of omega-6 and omega-3 PUFAs in the equine diet and its potential influence on inflammation. To the best of our knowledge there has been no research probing the effects of essential fatty acids on the equine skeletal system. Therefore, such an investigation is warranted and necessary to better understand the role these essential fatty acids play in biological pathways of the horse. The objectives of the present study were:

1) Investigate the effect of dietary omega-3 PUFAs from two sources on bone biochemical markers in young, rapidly growing horses

2) Evaluate fat supplemented diets and compare any influence on sub-clinical inflammation in young, exercising horses

3) Evaluate different dietary ratios of omega-6: omega-3 PUFAs in the equine diet and their composition in the blood

CHAPTER II

REVIEW OF LITERATURE

Bone Development In The Growing Horse

Proper bone development is essential in the young performance horse to ensure the skeletal structure of the animal will remain sound throughout training and competition. The physical demands of training submit these animals to excessive biomechanical strain; an undesirable condition impressed on an immature bone structure. Studies evaluating juvenile Thoroughbreds in training listed structural unsoundness as the primary causes of training failure (Bailey et al. 1999, Boston and Nunamaker 2000, Hernandez and Hawkins, 2001, Wilsher et al., 2006).

In a physiologically normal, mature horse, bone activity remains constant throughout its lifespan. In the growing horse, bone formation exceeds resorption, resulting in skeletal hypertrophy. Bone modeling involves the activation of osteoblasts, mononuclear bone cells which function to deposit mineral; adding shape, width and length to the bone matrix. During this phase, approximately 100% of the bone surface is active to achieve such a dynamic change (Watkins et al., 2001a). This significant increase in bone mass only occurs in the young horse, while bone remodeling is always occurring to some extent. Bone remodeling is the coupled process of resorption and formation. Under normal circumstances, there is no net loss or gain of bone. There are six phases of remodeling: quiescence, activation, resorption, reversal, formation and a return to quiescence. During both quiescence phases, osteoblasts are dormant and are laying flat along the bone surface.

Osteoclasts are multinucleated cells whose function is to break down the bone matrix resulting in the release of such minerals as calcium and phosphorous into the blood stream. Initiation of bone resorption occurs when mechanical loading, insult to the limb or abnormal metabolic activity stimulates a hormonal response which activates pre-osteoclastic cells.

During the resorption phase, osteoclasts are responsible for the removal of bone, leaving indentations within the matrix known as Howship lacunae. The amount of bone removed is dependent upon the volume and activity level of the osteoclasts (Sampson, 2005), with an increase in activity leading to an increase in bone resorption. Osteoclasts presence begins to decline with the subsequent appearance of new osteoblasts into the Howship lacunae during the reversal phase. Formation involves the synthesis of bone matrix and mineralization orchestrated by the presence of the new osteoblasts. Again, the degree of formation is dependent upon the amount of mononuclear cells and their contributing activity (Sampson, 2005) assuming all processes are undergoing normal metabolic functions. Mineralization of mature bone is approximately 76% in equines at one year of age (Lawrence, 2003) with approximately 50% of the primary equine skeleton being replaced, through remodeling, by three years of age (Marlin and Nankervis, 2002). In short; equine bone is extremely dynamic and active, with a short window of opportunity in which to maximize bone density and strength.

It has been well documented that weight-bearing activity is necessary to stimulate bone activity as well as maintain bone integrity and health. The conception that bone will adapt to environmental stress by altering its shape and composition is known as Wolff's law; early work by Woo and colleagues (1981) validate the concept in swine. Some form

of mechanical loading of the bone is required in order for bone density to increase in volume, thus providing a more stable foundation in which to accommodate impact. This concept has been thoroughly investigated and validated by a number of equine researchers (Nielsen et al., 2002, Hiney et al., 2000, Hoekstra, et al., 1999, Bell, et al., 2001). The degree and duration of physical activity necessary to achieve optimal bone growth and density has also been investigated and substantiated (Nielsen et al., 1997, Buckingham and Jeffcott, 1991, Jeffcott et al., 1987) with certain intensities being specific for the type of activity the horse is engaged. Hiney (2004) presented reviewed literature on bone development in horses, indicated that the age of the animal is crucial for bone development; as the juvenile animal is better suited for bone adaptation to exercise than the mature animal. For the purpose of the present study, the exercise protocol was designed to mimic the normal pasture activity of rapidly growing yearlings.

Omega 3 and Omega 6 Polyunsaturated Fatty Acids

Research in human nutrition has indicated how the supplementation of particular fats in the diet can have beneficial effects on inflammation and bone metabolism. The particular fats mentioned are the class of omega 3 fatty acids, found in the largest quantities in marine fish oil. Typical horse diets consist of the addition of plant-based oils such as corn or safflower. However these tend to be high in the omega 6 fatty acids, mainly Arachidonic acid (AA). This fatty acid is the parent of inflammatory responders known as prostaglandins, leukotrienes and thromboxanes. These compounds are necessary in the inflammatory response; however, if large concentrations are circulating in the system, the inflammatory mechanism can be exacerbated.

During lipid metabolism, the most common long chain omega-6 fatty acid is arachidonic acid (AA) while the most common long chain omega-3 fatty acid is eicosapentaenoic acid (EPA). Arachidonic acid derivatives include PGE_2, thromboxane A_2 (TXA_2) and leukotriene B_4, all part of a family of very effective signaling molecules responsible for inflammation and platelet aggregation. Eicosapentaenoic acid derivatives, prostaglandin H_3 (PGH_3) and thromboxane A_3 (TXA_3) are regarded as less potent substrates when compared to AA derivatives. Lee et al., (1991) commented that EPA competes with AA for metabolism by the cyclooxygenase and lipoxygenase pathways; therefore when available in adequate quantities, EPA can depress the production of the pro-inflammatory metabolites of AA, through its own production of the less inflammatory eicosanoids. Excess AA can lead to the increase in the formation of Prostaglandin E_2, its ω-6 derived eicosanoid. In adequate levels, PGE_2 is instrumental in many biological processes; however, excess amounts can be detrimental, promoting unnecessary bone resorption (Watkins et al., 2000) and exacerbating inflammatory responses (Calder, 2002).

One of the premier sources of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) is marine fish oil, also known as menhaden oil. Several studies have used menhaden oil as a supplement; however there is often difficulty when trying to incorporate it into equine diets due to poor palatability.

Eicosapentaenoic acid, though found in the largest quantities in marine fish oil, originates from alpha linolenic acid (ALA) which is converted from linolenic acid (LA) in plants. Plant oils such as linseed, soybean and flaxseed oils are all good sources of ALA, therefore, when consumed; the animal should be able to synthesis EPA and DHA

from these plant oils. In the use of the plant source of omega-3 fatty acids, EPA has been documented to inhibit the synthesis of AA from LA, decreasing inflammatory and procoagulant responses (Henry et al., 1990). One study involving western performance horses concluded that feeding horses a soybean oil diet did not increase fibrinogen concentration (an indicator of inflammation) when compared to horses fed a non-fat supplemented diet. Additionally, those horses fed the omega-3 diet appeared to recover quicker from inflammation when rested compared to horses fed an elevated omega 6 diet. (Wilson et al., 2003). Another study comparing corn oil to menhaden oil concluded that mean PGE_2 production was greater in horses fed a corn oil diet when compared to the horses fed a fish oil diet over a 12 week period (Hall et al., 2004).

Diets containing increased levels of omega 3 PUFA have been shown to regulate inflammatory cytokine production in both arthritic human subjects (Kremer et al., 1987) and healthy men and women (Endres et al., 1989, Meydani et al., 1991) after at least 4 weeks of diet change. Other studies using feline and canine subjects concluded that diets enriched with omega-3 PUFA appear to suppress cell-mediated immune response (Wander et al., 1997) and alteration in platelet aggregation (Saker et al., 1998), respectively.

Dietary fat sources that exert potent biological roles on the skeletal tissues belong to the omega 6 and omega-3 families of essential fatty acids; with their derivatives of prostaglandins, cytokines and insulin-like growth factors influencing skeletal biology (Watkins et al. 2001a). Feeding varying ratios of omega-6: omega-3 fatty acids have been shown to alter PGE_2 production in bone, leading either an increase in bone formation or a decrease in bone degradation (Korotkova et al. 2004; Requirand et al.

2000; Watkins et al. 2000). It appears that these essential fatty acids may also influence calcium absorption in animal subjects (Claassen et al., 1995, Kruger et al., 1997, Kelly et al., 2003) as well as blood calcium levels in horses (Hall et al., 2004).

Bone Biochemical Markers

A non-invasive method in evaluating bone activity is the use of bone biochemical markers. This practice has been utilized for years by human researchers and has been readily accepted into equine research in the past decade. The use of systemic markers identifies overall bone health; however it lacks specificity in identifying abnormalities in certain bones. Biochemical markers are ideal for 'real-time' assessment of bone activity and are useful for short-term evaluation, where as radiography provides a more unambiguous appraisal of bone health, providing a more specific identification of atypical bone.

Osteocalcin (OC) is a vitamin-K dependent, calcium binding protein (Price et al., 1976) produced by active osteoblasts during formation. Intacted remnants of this non-collagenous protein are excreted into circulation, where measurable increases in the blood indicate bone formation. Osteocalcin however does not reflect true osteoblastic activity, as the protein is incorporated into the new matrix as well as released during degradation (Kleerekoper, 1996). Therefore, OC is a better indicator of total bone activity rather than a gauge of bone formation. The use of OC as a bone biochemical marker has been used as a measure of bone turnover in both juvenile (Lang et al., 2002, Fenton et al., 1999) and mature horses (Price et al., 1995, Black et al., 1999). There appears to be a diurnal variation in serum OC levels with the highest concentrations being seen in the early morning hours (0200h-0900h) with peak levels occurring at approximately 0500h

(LePage et al., 1991, Black et al., 1999). Consequently, care must be taken relative to the time of day samples are collected.

During bone turnover, osteoclasts work to breakdown mineral as well as the collagen structure that makes up 90% of the bone matrix. The carboxy-terminal telopeptide of type I collagen (ICTP) is released into the blood during degradation with its concentration being subsequently measured in serum samples. An assay developed in 1993 detects ICTP as a marker of bone degradation (Risteli et al., 1993) and such methods have been used and validated in a number of investigations involving horses (Hiney et al., 2000, Michael et al., 2001, LePage et al., 1998, Price et al., 1995).

Acute Phase Response

Modifications to cellular metabolism and function due to excessive exercise, heat stress, tissue trauma or exposure to viruses contributes to a process known as "acute phase response". This is an early and immediate response within the inflammation process. Physical, environmental and internal stresses stimulate the reaction of monocytes and macrophages by initiating local and systemic changes. Early in the acute phase response, the events remain localized at the site of injury. This results in the identification of any loss of vascular integrity demarcate damaged tissues and recruit cells for the subsequent reparative phase, which involves a systemic response (Blok et al., 1996). Initial events involve macrophages or monocytes that stimulate the release of certain cytokines, in particular, interleukin-1 (IL-1) and tumor necrosis factor (TNF). IL-1 and TNF trigger additional cells to release pro-inflammatory prostaglandins, such as PGE_2, that works to modulate immune functions during inflammation. These particular mediators create alterations such as induction of fever, vasodilatation resulting in tissue

edema, and changes in the metabolism and gene expression within the liver. The liver is the target organ of systemic inflammatory regulation and is responsible for identifying the type of metabolite needed to support the animal during critical stages of stress (Baumann and Gauldie 1994). Hepatic contribution to inflammation involves decreasing the production of conventional plasma proteins, such as albumin and transferrin, while increasing the production of acute phase proteins such as serum amyloid A, ceruloplasmin and fibrinogen.

The acute phase response has been measured in horses by use of inflammation models such as the tissue-cage model (Higgins and Lee, 1984a), polyester sponge model (Higgins et al., 1987) and subcutaneously placed tissue chambers (Guthrie et al., 1996). These studies all used a carrageenan-induced method of inflammation with observed increases in either cytokine, prostaglandin or acute phase protein concentrations. Intramuscular injections of Freund's adjuvant into the neck of horses were successful in initiating an inflammatory response (Mills, et al., 1997; Auer et al., 1989). These are all excellent methods in which to evaluate the inflammatory response, however, there is limited research involving exercise-induced inflammation in horses. As a result, there is a great need to better understand how horses respond to training and to evaluate possible suppressors of inflammation generated through exercise.

Fibrinogen

One type of acute phase response protein is fibrinogen. Synthesized by the liver, fibrinogen's primary occupation is system hemostasis. Through the activity of thrombin, fibrinogen is cleaved into fibrin, an insoluble fibrous protein that required in the coagulation cascade and subsequent clotting mechanisms that occur post-injury. Horses,

compared to other species, are efficient in producing fibrinogen as a result of trauma, therefore, determining fibrinogen concentrations and measuring its change is a reliable indicator of inflammation (Andrews et al., 1994). Evaluation of fibrinogen levels is relatively simple, due in part to a method developed in 1971 (Millar et al.) and subsequently validated in horses by Campbell and associates in 1981. The procedure has been adopted for use in clinical settings due to its simplicity and has been an acceptable assay in equine research as well.

Normal levels of fibrinogen in horses have been identified as between 200-400 mg/dl, with values greater than 400 mg/dl regarded as a marker of inflammation. Increases in mean plasma fibrinogen above baseline values indicate the presence of acute phase response in horses (Mills et al., 1997, Schalm, 1979) with the degree of increase appearing to be related to the severity of injury (Allen and Kold, 1988). Observations of plasma fibrinogen conclude that fibrinogen concentrations increase immediately once acute phase response is initiated with peak levels occurring around d 6 and remaining elevated for several days (Allen and Kold, 1988, Mills et al., 1997, Wilson, et al., 2003). It should be noted that fibrinogen concentrations are also an indicator of inflammatory resolution, with concentrations decreasing immediately after peak values and ultimately returning to baseline values within a reasonable timeframe.

Prostaglandin E_2

Prostaglandins are a part of a family of potent eicosanoid lipid mediators derived from the cellular membrane bound n-6 fatty acid arachidonic acid (AA). AA is stimulated by phospholipases to be released from the cell and by interaction of cycloxygenase enzymes; yield eicosanoids. The exemplary characteristics of "true" eicosanoids are their

sterochemical precision in formation and recognition of their target tissues, effectiveness in the nanomolecular range in vitro and their authentic involvement in biological activities (Funk, 2001). The predominate prostaglandin found in most mammalian cells is prostaglandin E_2 (PGE_2), an important arbitrator of several biological processes, particularly immune response. PGE_2 is produced by several classifications of mammalian cells, such as macrophages, mast cells and fibroblasts, indicating that evaluation of their concentrations would provide a good interpretation of their involvement in bone activity and immune response.

Quantifying measurement of PGE_2 concentrations in horses is limited, with a majority of available information based on *in vitro* studies with either equine macrophages (Hall et al., 2004), equine articular chondrocytes (Munstaman et al., 2005) or equine synovial explants (Bertone et al., 2001). However, all fore mentioned inquiries were able to elevated PGE_2 concentrations above baseline when an inflammatory stimulator was introduced, verifying the presence of PGE_2 during the inflammatory response in the horse. PGE_2 concentrations varied depending on source of samples; normal synovial fluid PGE_2 concentrations were established at approximately 9.5 pg/ml, with levels exceeding 22 pg/ml indicating the presence of joint disease (Bertone et al., 2001). In vivo studies evaluating PGE_2 concentrations in tissue fluid established normal mean values at 2439 $^{+/-}$ 937 pg/ml (Guthrie et al., 1996) and 2900$^{+/-}$300 pg/ml (Higgins and Lee, 1984a). When stimulated, PGE_2 concentrations peaked on average at 27,572 $^{+/-}$ 9607 pg/ml (Guthrie et al., 1996) and 197,000$^{+/-}$ 6490 pg/ml (Higgins and Lee, 1984a) respectively.

There is little available information on the quantity of PGE_2 in equine blood and alteration in levels occurring during the acute phase response. Previous measurements taken after exercising horses revealed no detectable concentrations of PGE_2 (Mitten et al.,1995), however authors concluded that levels were most likely below the limits of sensitivity (50 pg/ml) for the assay they were using. Investigation of eicosanoid levels in normal horses specified mean digital vein PGE_2 concentrations to be 187.18 pg/ml under resting conditions (Owens, et al., 1995). Further queries of plasma PGE_2 levels in exercising horses are necessary to better understand its involvement during exercise-induced inflammation.

CHAPTER III

MATERIALS AND METHODS

Animals and Background Period

Nine yearling Quarter Horses were blocked by gender and age and randomly assigned to a triplicated 3X3 Latin square design of diet treatments. Horses averaged 352 kg in body weight and were approximately 14 months of age at the start of the experiment. Prior to beginning the pre-trial period all horses were evaluated for health status. The horses were placed on a routine deworming, inoculation and feet trimming schedule. Horses were maintained at the Texas A&M University Horse Center following management protocol as stated in the Animal Use Protocol approved by the Institutional Agricultural Animal Care and Use Committee.

A 14 day pre-trial conditioning period was conducted prior to the beginning of the experiment to achieve a comparable level of fitness and to achieve a baseline level of physiological effects of exercise among all horses. Yearlings were accustomed to a mechanical freestyle equine exerciser prior to the beginning of the experimental periods. The equine exerciser (Summertree Co., Inc.) is designed with an interior track consisting of six individual paneled sections, allowing horses to be exercised freely without being tied to the apparatus. Exercise on d 1 consisted of a total of 10 minutes of walking and 10 minutes of trotting. Exercise adaptation on d 2 involved a total of 7 minutes of walking and 17 minutes of trotting. By the third day in the freestyle exerciser, horses were walking for a total of 10 minutes, trotting for 13 minutes and loping for 8 minutes. By d 4 horses were walking for 7 minutes, trotting for 18 minutes and loping for 6 minutes. On d

5 of background exercise, yearlings were walking for a total of 8 minutes, trotting for a total of 16 minutes and loping for a total time of 8 minutes. Exercise on d 6-8 had the horses walking for an average of 6 minutes, trotting for an average of 13.5 minutes and loping for approximately 10 minutes. All times for the various gaits indicated were equally divided throughout each exercise session, with a change of direction occurring half way through the session to allow for equal loading of each front limb.

Experimental Diet

Each period involved 28-d of diet adaptation and exercise protocol. Diets consisted of a formulated concentrate containing either 8% corn oil (CO), 8% mechanically extracted soybean oil (SBO), or a 3:1 ratio corn: menhaden oil[1] mix with volume equaling 8% of the diet (MCO) (Table 1). Oils were measured out for each individual horse and kept refrigerated (4° C) until they were topdressed on concentrates during feedings. Yearlings were fed a coastal Bermuda grass hay to meet fiber and additional nutrient requirements. Diets were formulated to be isocaloric and based on the NRC requirements (1989) for yearlings undergoing rapid growth and according to recommendations by Nielsen et al. (1998) and Michael et al. (2001). Concentrate was formulated by authors, though mixed and bagged by Producers Cooperative, Bryan, Texas. Table 2 shows the nutrient composition of the three diets. The total diet was comprised of 52% extruded concentrate, 8% assigned oil, and 40% coastal Bermuda grass hay.

[1]Omega Equis, Omega Protein Inc. Houston, TX .

Table 1. Composition of total diet (%; as fed)

Diet Composition	
	%
Forage	40
Oats	15.73
Corn	19.24
Soybean Meal	13
Salt	0.26
Molasses	2.08
Dical	0.52
Vit/Min premix	0.26
Limestone	0.91
Oil	8
Total	100

Table 2. Calculated composition of diets (as fed)

Component	Corn Oil Supplemented Diet	Menhaden/Corn Oil Supplemented Diet	Soybean Oil Supplemented Diet
Digestible Energy			
Mcal/kg	3.15	3.16	3.15
Crude Protein %	13.44	13.44	13.44
g/Mcal	42.67	42.53	42.67
Crude Fat %	10.56	10.56	10.56
g/Mcal	33.52	33.42	33.52
Ca %	0.70	0.70	0.70
g/Mcal	2.22	2.22	2.22
P %	0.35	0.35	0.35
g/Mcal	1.11	1.11	1.11
Mg %	0.16	0.16	0.16
g/Mcal	0.51	0.51	0.51
K %	0.90	0.90	0.90
g/Mcal	2.86	2.85	2.86

Horses were housed in groups of three in 9 X 9 meter dry lot pens with water offered ad libitum. Horses were individually fed their designated diets in two equal feedings per day at 0600h and 1800h. Initial ration amounts were based on approximately 2% BW of each individual horse with rations adjusted as needed to maintain a body condition score between 5 to 6 (Henneke et al., 1983).

Experimental Exercise Protocol

On d 1- 5 of each week horses were subjected to a standardized exercise protocol consisting of 2 minutes of walking, 3 minutes of trotting, 30 seconds of walking, 4 minutes of loping, 1 minute of walking, 3 minutes of loping, 1 minute of walking, 3 minutes of loping, 2 minutes of walking, trotting for 2 minutes and finally a warm down of 3 minutes of walking. Horses changed direction during each exercise session after either the first or second lope, preventing excessive loading to any one particular limb.

Measurements and Data Collection

On d 0, 28, 56 and 84 of the experiment physical measurements were taken to evaluate growth. Body weight and body condition scores were taken as needed throughout the experiment with feed rations adjusted if needed to maintain body condition. Physical measurements included body weight, wither height, hip height, forearm circumference, gaskin circumference and heart girth. On each measurement day, sample spots on the forearm, gaskin, point of the shoulder and point of the hip were clipped and measurements were taken from selected points within the clipped area. Subsequent measurements were taken from designated sample spots to minimize error.

Fasting blood samples were taken from all horses prior to morning feeding on d 0, 3, 7, 10, 14, 17, 21, 24 and 28 of each period via veni-jugular puncture. All blood

collections occurred at approximately 0500h in accordance with findings by Black et al. (1999) indicating the diurnal variation in bone biochemical markers.

Blood serum samples were harvested into single 10 ml blood collection evacuated tubes without additives. Venous blood samples for plasma were collected into duplicate 10 ml blood collection evacuated tubes containing lithium heparin. Additional blood was collected into single 5 ml evacuated tubes containing EDTA. One vial of the heparinized blood from each horse was kept refrigerated (4°C) for immediate fibrinogen analysis. All other blood collection tubes were spun at 3,200 rpm in a balanced centrifuge at 4°C for 30 minutes. After separation, plasma or serum from each tube was pipetted into labeled 1.5ml microcentrifuge tubes, properly capped and stored upright in Styrofoam carriers at -20°C until needed for analysis.

Concentrate samples, oil samples and forage samples were taken at random throughout experimental periods for future analysis of fatty acid content and nutritional composition.

Laboratory Analyses

Dry matter content of concentrate and forage samples were conducted. Feed samples were ground using a Wiley mill accommodated with a 1 mm mesh screen. Approximately 2.0 g of ground concentrate and forage sample were measured in triplicate into aluminum pans and placed in a drying oven (68°C) for 72 hours. Pans were removed and placed inside a dessicator for cooling and to prevent condensation. Dry sample weight was determined by subtracting sample weight prior to drying from the sample weight after drying. Dry matter percentage was calculated using the following formula:

$$\frac{\text{Dry sample weight (g)}}{\text{Wet sample weight (g)}} \text{ X } 100$$

Samples were reanalyzed if standard error was greater than 5%.

Plasma fibrinogen concentration of blood samples were assayed within 24 hours of blood collection. Fibrinogen concentration was determined using micro-hematocrit heat precipitation methods of Millar et al. (1971) and Wilson et al. (2003). Whole blood was harvested into blood collection tubes containing heparin. Two 75 mm heparinized micro-hematocrit capillary tubes (Fisher Scientific) were filled at least 50% with whole blood and sealed at one end using critoseal (Oxford Labware). The tubes were centrifuged for 5 minutes. Tubes were immediately transferred upright into a water bath (56 +/-2°C) for 3 minutes, centrifuged for an additional 3 minutes. Using a digital caliper, the length of the fibrinogen column and total length of fibrinogen-plasma column was measured. Fibrinogen concentration (mg/dl) of each sample was determined using the following equation:

$$\frac{\text{Fibrinogen column}}{\text{Fibrinogen-plasma column}} \text{ X } 10{,}000$$

Plasma samples, oil samples, concentrate samples and forage samples were analyzed for total fatty acid profiles using a gas chromatograph. Heparized plasma samples were thawed, with 3 ml of plasma pipetted into 55 ml glass centrifuge tubes. 400 µl of oil samples; and 1 g of dried ground concentrate and forage samples were placed in their respective 55 ml glass centrifuge tubes. 15 ml of a 2:1 solution of $CHCl_3$: CH_3OH was added to tubes and mixture was vortexed for 30 sec. The homogenate was pipetted out and filtered through a Whatman filter apparatus using a 2.4 cm GF/C filter into a

second labeled 55 ml glass centrifuge tube. The first tube and the filter funnel were rinsed three times with $CHCl_3$: CH_3OH solution and filtered into a second labeled 55 ml glass tube. Enough $CHCl_3$: CH_3OH solution was then added to each filtered homogenate sample to make the volume approximately 30 ml after which 8 ml of 0.74% KCl was added. Tubes were vortexed for 1 minute. Sample tubes were capped with nitrogen and stored (-4ºC) overnight in cooler to allow homogenate to separate into two separate phases. Once phase separation occurred, the upper phase was carefully removed using a filter flask vacuum apparatus. The lower phase was transferred to clean, labeled 20 ml glass tubes. The 55 ml glass tubes were rinsed three times with $CHCl_3$: CH_3OH solution and transferred into the 20 ml glass tubes that contained the lower phase. Samples were then placed into a Meyer N-Evap analytical evaporator to allow for complete evaporation of $CHCl_3$: CH_3OH solution, leaving only the dried lipid content of the original plasma sample. After evaporation, 1 ml of 0.5 N KOH in MeOH was added to each sample; which was loosely capped, and transferred to a water bath (70°C) for 10 min. 1 ml of 14% BF_3 in MeOH is then added to each sample. Tubes were then flushed with nitrogen, loosely capped and placed back into the water bath (70°C) for 30 min. Tubes were removed and allowed to cool to room temperature. 2 ml of GC-grade hexane and 2 ml of saturated NaCl was added to the samples. Tubes were vortexed again for 30 sec. Upper hexane layer was removed and transferred into labeled 20 ml glass tubes containing ~800 mg Na_2SO_4. An additional 2ml of hexane were added to saturated NaCl tubes and briefly vortexed. The upper hexane layer was then transferred into the Na_2SO_4 tubes, which now contained ~ 4ml of hexane, and briefly vortexed. Upper hexane layer was transferred into labeled scintillation vials. 1 ml of hexane was added to Na_2SO_4 tubes, and briefly

vortexed. Remaining upper hexane layer was transferred into labeled scintillation vials. Scintillation vials were then transferred to N-Evap to allow hexane to evaporate completely. Lipids in scintillation vials were reconstituted with 0.5 ml of hexane and mixed carefully. Oil samples were processed identical to plasma and feedstuff samples, with the exception of being reconstituted with 1 ml of hexane. 100µl of sample solution was pipetted from scintillation vials into labeled 2 ml autosampler vials. Autosampler vials were then loaded into a Varian Chrompack automated gas chromatograph for analysis. Lipids in scintillation vials were reconstituted with .5 ml of hexane. Oil samples were reconstituted with 1 ml of hexane. 100µl of sample solution was pipetted from scintillation vials into labeled autosampler vials. Autosampler vials were then loaded into a Varian Chrompack automated gas chromatograph for analysis.

Serum was assayed for concentration of the bone formation marker Osteocalcin using an enzyme immunoassay kit (Quidel Corporation, San Diego, CA). Osteocalcin concentration was measured from serum samples obtained on day 0, 14 and 28 of each study period. Serum samples were diluted 10-fold with saline solution according to modifications validated by previous research (Lang et al., 2002).

Additional serum samples were analyzed for the concentration of the carboxy-terminal telopeptide of type I collagen (ICTP). Samples obtained on day 0, 14, and 28 of each period were assayed using a radioactive immunoassay (DiaSorin Inc., Stillwater, MN).

Prostaglandin E_2 (PGE_2) was analyzed from plasma samples collected on day 0, 3, 10, 17 and 24 of each period. Sample collections days were different from bone biochemical markers due to the days that weekly exercise commenced on. Blood was

drawn at the end of each week to avoid the weekend layoff causing variability in the inflammation measurement. An enzyme-linked immunoassay (R&D systems, Minneapolis, MN) was used, with all plasma samples requiring a 5-fold dilution with a provided calibrator diluent prior to beginning the assay.

All laboratory assays were run in duplicate unless otherwise noted and were reanalyzed if the standard error was greater than 5%.

Statistical Analysis

Data was analyzed for treatment, day and treatment-day-interaction by analysis of variance for Latin square using Stata Software (Stata Corp. 2004. College Station, Texas). Period interaction was originally evaluated in all analysis of variance tests; however, if period interaction was not observed, the variable was dropped to allow for a stronger statistical model. In order to evaluate change within periods, hematological data was normalized to day 0 of each period, unless otherwise indicated. When main differences were observed at $P < 0.05$ level, means were further separated using the Bonferroni means comparison test.

CHAPTER IV

RESULTS

Feed Analysis

The fatty acid profile of the concentrate is shown in Table 3. The concentrate contained 3.68% crude fat, with the relative percentage by weight of individual fatty acids in the concentrate to be 15.71% for C16:0 (palmitic acid), 32.69% for C18:1n-9 (oleic ccid), 39.15% for C18:2 (linoleic acid) and 1.79% for C18:3 (alpha linolenic Acid). There were scant readings of C20:5 (eicosapentenoic acid) and C22:6 (docosahexanoic acid); however they were less than 0.5%. The n6:n3 ratio of polyunsaturated fatty acids of the concentrate was 18.41.

The fatty acid profile of the forage is shown in Table 4. The Coastal Bermudagrass hay contained 1.63% crude fat with the relative percentage by weight fatty acids identified being 23.20% C16:0 (palmitic acid), 4.69% C18:1n-9 (oleic acid), 14.81% C18:2n-6 (linoleic acid) and 20.38% C18:3 (alpha linolenic acid). The forage contained small amounts of C20:5 (EPA) and C22:6 (DHA) at 0.39% and 0.84% respectively. The n6:n3 ratio of polyunsaturated fatty acids found in the forage was 0.77.

Table 3. Fatty acid profile of concentrate

Fatty Acid	% by weight
C14:0	0.13
C16:0	15.71
C16:1n-7	0.16
C18:0	2.77
C18:1n-9	32.69
C18:2n-6	39.15
C18:3n-3	1.79
C20:4n-6	0.21
C20:5n-3	0.27
C22:6n-3	0.07
n6:n3 ratio	18.41

Table 4. Fatty acid profile of Bermudagrass hay

Fatty Acid	% by weight
C14:0	1.02
C16:0	23.19
C16:1n-7	0.1327
C18:0	3.43
C18:1n-9	4.69
C18:2n-6	14.81
C18:3n-3	20.38
C20:4n-6	2.02
C20:5n-3	0.39
C22:6n-3	0.83
n6:n3 ratio	0.77

The chemical fatty acid analysis for corn oil is shown in Table 5. Relative percentage by weight of fatty acids identified within corn oil were 11.86% C16:0 (palmitic acid), 26.79% C18:1n-9 (oleic acid), 55.85% C18:2n-6 (linoleic acid) and 0.98%C18:3 (alpha linolenic acid). There were non-detectable measurements of either C20:5n-3 (EPA) or C22:6n-6 (DHA) in the corn oil. The n6:n3 polyunsaturated fatty acid (PUFA) ratio of corn oil was 56.97. Corn oil was added to the concentrate in the amount of 8% of the diet. The n6:n3 polyunsaturated fatty acid ratio for the total CO diet was calculated to be 3.22:1.

Table 5. Fatty acid profile of corn oil

Fatty Acid	% by weight
C14:0	0.51
C16:0	11.85
C16:1n-7	0.44
C18:0	2.24
C18:1n-9	26.78
C18:2n-6	55.85
C18:3n-3	0.98
C20:4n-6	ND*
C20:5n-3	ND*
C22:6n-3	ND*
n6:n3 ratio	56.97

*denotes non-detectable presence of interested fatty acid

Corn oil and Menhaden oil was mixed at a 3:1 ratio with volume equaling 8% of the total diet. Chemical fatty acid profile of the mix is shown in Table 6. When analyzed, menhaden/corn oil mix had a fatty acid profile (% by weight) of 12.85% C16:0 (palmitic acid), 22.69% C18:1n-9 (oleic acid), 43.02% C18:2n-6 (linoleic acid) and 1.17% C18:3 (linolenic acid). Measurements of C20:5 (EPA) and C22:6 (DHA) were considerable at 3.09% and 3.38%, respectively. Calculated PUFA n6:n3 ratio of the menhaden/corn oil 5.64 :1. The n6:n3 polyunsaturated fatty acid ratio for the total MCO diet was calculated to be 2.96:1.

Table 6. Fatty acid profile of menhaden/corn oil 3:1 Mix

Fatty Acid	% by weight
C14:0	1.82
C16:0	12.85
C16:1n-7	2.49
C18:0	2.43
C18:1n-9	22.69
C18:2n-6	43.02
C18:3n-3	1.17
C20:4n-6	0.16
C20:5n-3	3.09
C22:6n-3	3.38
n6:n3 ratio	5.64

Results for mechanically-extruded soybean oil analyzed for fatty acid profile is shown in Table 7. Percentage by weight of specific fatty acids include 10.62% C16:0 (palmitic acid), 20.78% C18:1n-9 (oleic acid), 55.09% C18:2 (linoleic acid) and 8.08% C18:3 (alpha linolenic acid). There were no detectable measurements of either C20:5 (EPA) or C22:6 (DHA) present in the mechanically-extruded soybean oil.

Soybean oil alone contained a PUFA n6:n3 ratio of 6.81:1. The n6:n3 polyunsaturated fatty acid ratio for the total SBO diet was calculated at 2.96:1.

Table 7. Fatty acid profile of mechanically extruded soybean oil

Fatty Acid	% by weight
C14:0	0.07
C16:0	10.62
C16:1n-7	0.07
C18:0	4.17
C18:1n-9	20.78
C18:2n-6	55.09
C18:3n-3	8.08
C20:4n-6	ND*
C20:5n-3	ND*
C22:6n-3	ND*
n6:n3 ratio	6.81

*denotes non-detectable presence of interested fatty acid

Body Weight and Feed Intake

There were no significant difference in body weight among yearlings fed the different dietary treatments (P= 0.9876) (Table 8). Consumption of concentrate and hay were not statistically different between diets (P =0.6956) and (P =0.5940), respectively. Mean concentrate intake among all groups was 4.32 kg/d, with average forage intake among all horses approximately 2.85 kg/d. There were no palatability issues when oils were added to the concentrate. Any initial refusals of feed due to changing diets lasted no longer than one feeding.

Table 8. Body weight, concentrate intake, hay intake and total intake

	Treatment			
Component	CO	MCO	SBO	All Groups
Weight (kg)	370.76	369.83	368.72	369.77
SEM	19.18	21.71	20.15	11.54
Concentrate Intake (kg)	4.31	4.26	4.407	4.32
SEM	0.16	0.10	0.14	0.07
Hay Intake (kg)	2.83	2.83	2.89	2.85
SEM	0.10	0.06	0.09	0.05
Total Intake (kg)	7.14	7.09	7.297	7.18
SEM	1.05	1.01	1.07	0.03

Physical Measurements

Physical measurements taken on d 0 and d 28 of each period were analyzed for statistical differences among diets. Measurements did not differ among treatments for wither height, hip height, body length, heart girth area, forearm circumference or gaskin circumference ($P > 0.05$). Mean values for physical measurements are listed in Table 9.

Plasma Fatty Acid Profiles

Plasma samples acquired on d 0 and d 28 of each period were analyzed for identification of specific fatty acids. Percent by weight of plasma fatty acids identified on d 0 (baseline) are shown in Appendix 1. Mean basal fatty acid values (% by weight) were not different between groups ($P > 0.05$) and values were consistent with previous research evaluating equine plasma fatty acid profiles in yearling horses (Luther et al., 1981). The mean value of plasma fatty acids identified on d 28 with percent change in plasma fatty acids from baseline (d 0) is shown in Table 10.

Table 9. Influence of diet on parameters of growth

	CO	SEM	MCO	SEM	SBO	SEM
Weight, kg						
Initial	372.85	14.51	365.59	18.15	344.73	34.81
Final	398.56	12.06	378.29	28.11	404.91	8.18
Gain	32.96	8.92	33.56	7.25	32.05	7.63
Wither Height, cm						
Initial	133.56	1.38	136.74	1.84	139.06	3.72
Final	139.28	1.84	141.82	3.30	139.48	1.38
Gain	2.54	0	2.75	.56	5.92	2.77
Hip Height, cm						
Initial	142.45	1.28	143.72	.56	145.20	4.03
Final	146.05	.73	148.16	3.69	144.78	1.27
Gain	2.32	.76	2.96	.42	2.32	.56
Body Length, cm						
Initial	150.71	48.15	152.29	3.44	149.43	6.16
Final	152.08	5.30	155.25	4.86	156.11	1.16
Gain	-.21	4.21	5.82	1.78	5.39	.84
Heartgirth, cm						
Initial	163.19	2.28	162.56	3.86	158.43	6.20
Final	170.71	2.32	165.1	6.00	170.07	1.37
Gain	8.14	1.70	6.66	1.45	6.87	1.07
Forearm, cm						
Initial	48.15	1.60	51.14	1.10	48.57	2.52
Final	51.54	1.22	48.57	3.53	49.42	1.10
Gain	.39	.95	-3.97e-08	1.14	1.27	1.27
Gaskin, cm						
Initial	42.86	.66	43.49	.84	41.48	1.48
Final	44.87	1.48	42.55	1.14	43.18	.66
Gain	1.37	1.01	1.05	.38	.32	.36

Table 10. Mean plasma fatty acid profile on D 28 (% by weight) and percent change in fatty acid profile in response to dietary treatment

Fatty Acid	CO		MCO		SBO	
(% by weight)	Mean	SEM	Mean	SEM	Mean	SEM
C14:0	0.11	0.05	0.03	0.02	0.02	0.01
C14:0 (% Δ)	18.27	53.92	-69.80	18.98	-80.04	8.91
C16:0	9.59[a]	0.56	8.83[a]	0.74	7.70[b]	0.68
C16:0 (% Δ)	0.63	10.02	-8.59	9.36	-21.28	6.65
C16:1n-7	0.22[a]	0.02	0.20[a]	0.03	0.15[b]	0.02
C16:1 n-7 (% Δ)	-22.59[a]	9.01	-31.09[a,b]	9.01	-50.39[b]	8.24
C18:0	16.90[a,b]	0.65	16.07[a]	0.81	18.58[b]	0.95
C18:0 (% Δ)	-4.96	4.44	-9.65	5.05	4.35	5.72
C18:1	9.61	0.21	9.09	0.47	9.54	0.73
C18:1 (% Δ)	-5.84	3.59	-11.39	3.89	-3.21	11.93
C18:2	55.98	1.34	54.56	0.45	57.08	0.61
C18:2 (% Δ)	5.76	2.24	3.18	1.34	7.96	1.59
C18:3	0.39	0.04	0.26	0.09	0.40	0.06
C18:3 (% Δ)	4.37	40.82	-40.49	27.42	-9.15	36.75
C20:4	1.18[a]	0.05	1.78[b]	0.07	1.38[a]	0.09
C20:4 (% Δ)	-37.89[a]	4.12	-7.02[b]	4.74	-28.14[a,b]	4.41
C20:5	0.09[a]	0.09	1.36[b]	0.19	0.01[a]	0.01
C20:5 (% Δ)	101.05[a,b]	114.08	355.36[b]	140.33	-21.62[a]	14.82
C22:6	0.07[a]	0.03	1.64[b]	0.18	0.21[a]	0.05
C22:6 (% Δ)	-21.61[a]	16.91	678.98[b]	146.25	6.23[a]	28.01

[a,b]Rows not sharing common superscripts differ (P<0.05)

When horses were fed SBO, there was a significant decrease in plasma C16:0 (palmitic acid) and plasma C16:1n-7 (palmoleic acid) percent by weight over 28 days when compared to horses consuming either MCO or CO diets ($P<0.05$). Though numerical different, there were no difference in percentage change in C16:0 between diets ($P > 0.05$). Percent change in profiles of C16:1 were significantly lower in horses fed SBO diet when compared to horses consuming the CO diet ($P < 0.021$).

Horses fed the SBO diet had a significantly higher plasma profile (% by weight) of C18:0 (steric acid) compared to horses consuming MCO diets ($P < 0.05$), however, there were no statistical difference between diets in percent change from baseline C18:0 values ($P > 0.05$).

There were no differences in percent by weight measurements of myristic acid, oleic acid, linoleic acid or alpha linoleic acid among treatments. Linoleic acid values (% by weight) increased in all horses over 28 days, while myristic acid, oleic acid and alpha linoleic acid values all declined regardless of dietary treatment. Profiles (% by weight) of C20:4 (arachidonic acid) were significantly higher in MCO horses compared to horses being fed either SBO or CO diets ($P = 0.0000$). The menhaden oil provided an exogenous source of arachidonic acid, while both soybean oil and corn oil had non-detectable amount of arachidonic acid. The menhaden oil used in this study was 2.09% by weight arachidonic acid and when mixed with corn oil, MCO had an arachidonic cid % by weight of 0.16. However, the percentage change in arachidonic acid fatty acid profile from baseline fatty acid profile (% by weight) were only significant between MCO horses and CO horses ($P < 0.0032$), with C20:4 profile in SBO fed horses were similar to the other two diets ($P > 0.05$).

Percent change in C20:5 (EPA) and C22:6 (DHA) from basal profile measurements (% by weight) were significantly higher in horses fed the MCO diet compared to horses fed either CO or SBO ($P < 0.0001$). The MCO diet provided the sole source of EPA and DHA, with a significant level being identified in horses consuming the mixture over 28 days [1.36% (by weight) EPA and 1.64% (by weight) DHA]. MCO fed horses expressed a 3 fold increase in plasma C20:5 concentrations over 28 days, with changes in plasma profile being significantly different from horses supplemented with SBO ($P < 0.0338$). Though numerically different, plasma profiles (% by weight) of C20:5 between corn oil fed horses and horses consuming the menhaden/corn oil mix did not differ ($P > 0.05$).

MCO fed horses had the highest levels of plasma C22:6 (DHA), compared to MO and SBO fed horses ($P <0.0001$), with percent change from baseline being significantly higher in MCO horses compared to horses supplemented with either corn oil or mechanically extruded soybean oil.

Serum Carboxyterminal Telopeptide of Type I Collagen

Serum samples taken on d 0, d 14 and d 28 of each period were analyzed for the bone degradation marker carboxyterminal telopeptide of type I collagen (ICTP) . Mean ICTP concentrations; separated by treatment and day, are shown in Appendix 2 and illustrated in Figure 1.

Mean ICTP concentrations in exercising yearlings were not different among treatments ($P = 0.4810$) nor was there an effect of sampling day on circulating serum ICTP levels ($P = 0.8903$). All horses regardless of diets had decreases in ICTP

concentrations from d 0 to d 14. There appeared to be a numerical difference in ICTP levels between diets at d 28, however it was not statistically different (P = 0.5446).

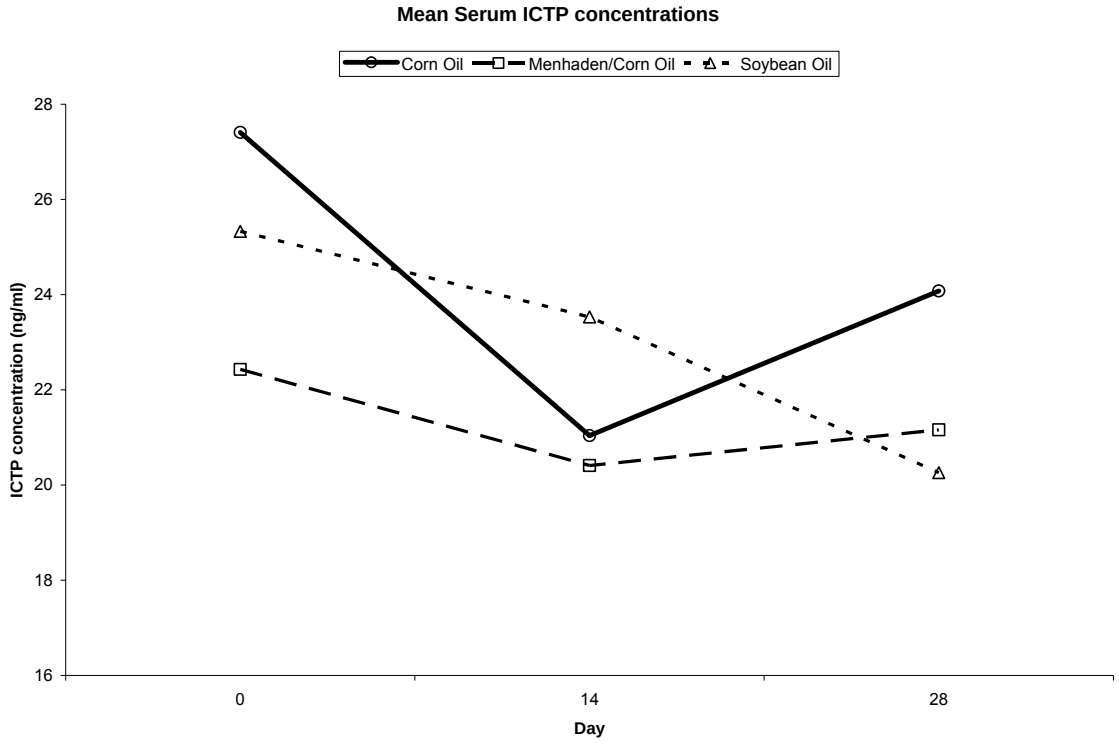

Figure 1. Mean ICTP concentrations by diet over a 28 day period

Due to large variation in ICTP levels among horses, data were normalized to d 0 of each period to monitor change over 28 days. Normalized ICTP concentrations, separated by treatment and day, are shown in Appendix 3 and illustrated in Figure 2.

There were no difference among treatments (P = 0.8887) or day (P = 0.8996) with normalized data. While not statistically significant, concentrations from horses consuming MCO diets appeared to drop sharply from d 0 to d 14 compared to the other diets, with a slight increase from d 14 to d 28. ICTP concentrations remained constant through d 14 then exhibited a rapid decrease to d 28. Horses fed CO diets appeared to

have numerically higher ICTP concentrations at d 28 than at d 0 however when d 28 were isolated and analyzed, there were no significant difference between treatments (P = 0.5628).

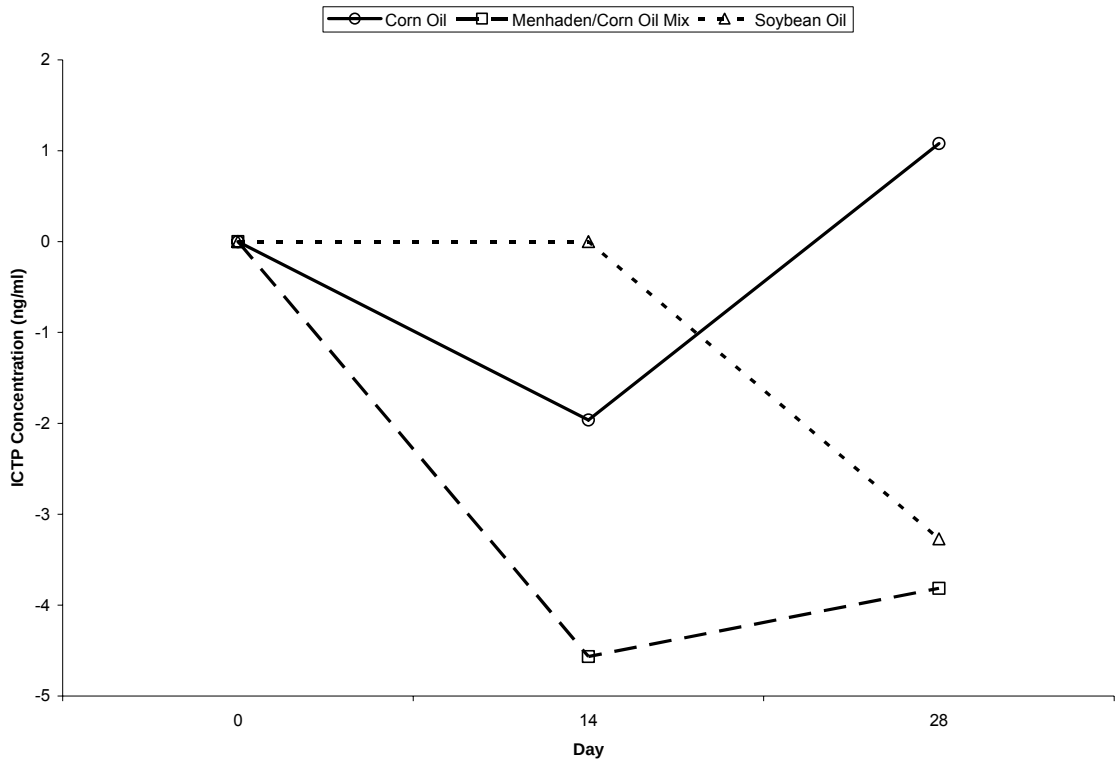

Figure 2. Normalized mean ICTP concentrations by diet over a 28 day period

Plasma Osteocalcin

Bone activity in yearling horses was evaluated by analyzing Osteocalcin (OC) concentration in plasma samples collected on d 0, d 14 and d 28 of each period. Mean OC concentrations, separated by treatments and day are shown in Appendix 4.

Based on mean unadjusted OC concentrations, there were no significant difference between treatments (P = 0.6865) or day within periods (P = 0.3119). Concentrations of OC among horses were not similar at d 0 (baseline) of the trial;

therefore values were adjusted to d 0 of each period and analyzed for change through d 28. Normalized OC data, separated by day and treatment, are shown in Appendix 5.

Normalized mean OC concentrations did not differ among diets (P = 0.4104), however there was a tendency for sampling day to effect OC measurements (P=0.1033). All Osteocalcin concentrations regardless of treatments numerically declined from d 0 to d 14, then increased between d 14 and d 28 (Figure 3). Horses fed MCO diets had the smallest numerical amount of change between d 0 and d 28, although there were no significance difference when compared to the other two dietary treatments (P>0.05) (Table 11).

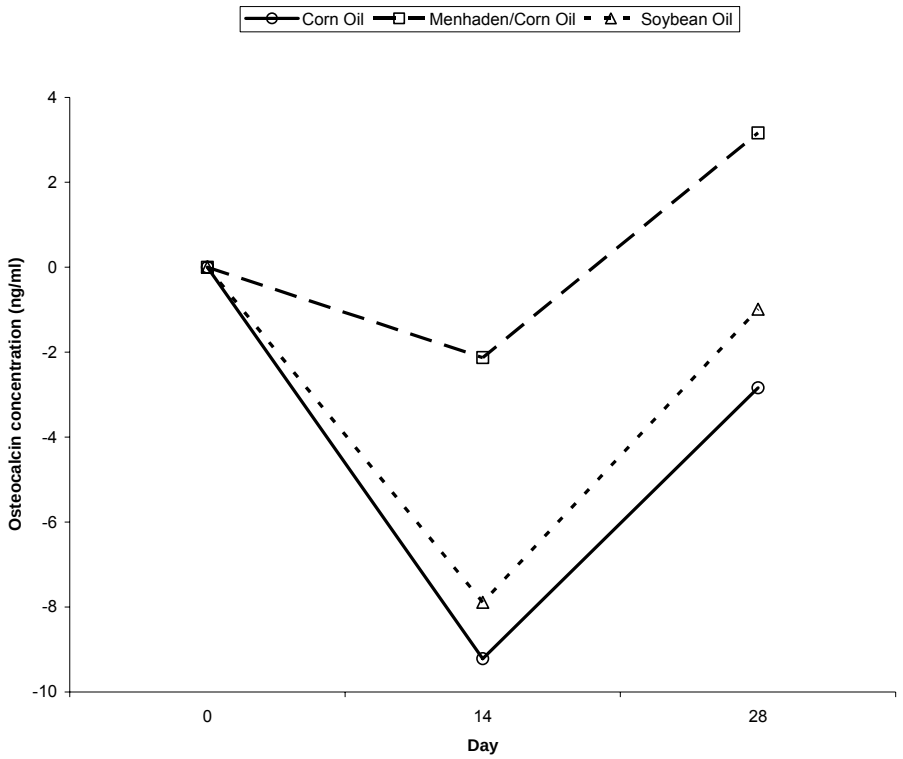

Figure 3. Normalized mean osteocalcin concentrations over a 28 day period

Unfortunately, there was a significant period effect with normalized OC data (P = 0.0353), with Osteocalcin concentrations decreasing over time from period 1 through period 3. (Table 12) Exercise was likely unsuccessful in stimulating a bone response during those time periods, resulting in a decrease in the bone marker.

Table 11. Normalized mean osteocalcin concentration (ng/ml) by diet and day

Day	0	14	28
CO (ng/ml)	0	-9.22	-2.83
SEM	0	6.07	4.63
MCO (ng/ml)	0	-2.12	3.16
SEM	0	4.4	6.28
SBO (ng/ml)	0	-7.89	-0.98
SEM	0	4.11	5.38

Table 12. Normalized mean osteocalcin concentration (ng/ml) by period

Period	1	2	3
OC (ng/ml)	1.89^{a}	$-1.57^{a,b}$	-6.94^{b}
SEM	2.57	2.75	1.63

^{a,b}Row means not sharing superscripts differ (P = 0.035)

Plasma Prostaglandin E_2

Prostaglandin E_2 (PGE_2) concentrations were established using plasma samples taken on d 0, d 3, d 10, d 17 and d 24 of each period. Mean PGE_2 values; separated by treatment and by day, are shown in Appendix 6.

For mean PGE_2 concentrations, there were no significant difference among treatments (P = 0.467). Though there is a numeric difference between diets at day 17

(Figure 4), there was no significant day effect (P = 0.816) on mean PGE_2 concentrations in exercising yearlings.

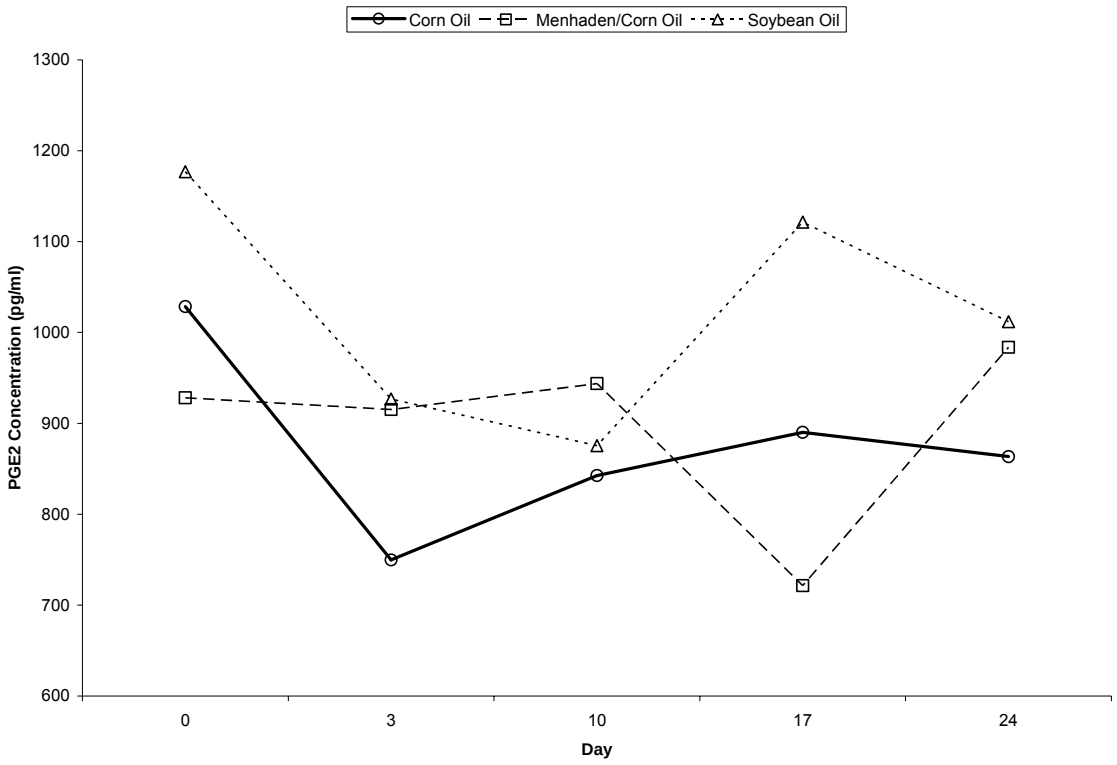

Figure 4. Mean PGE_2 concentration by diet

PGE_2 data were normalized to d 0 measurements of each period. There were no significant day effect (P = 0.661) or treatment influence (P = 0.421) on normalized PGE_2 production among yearlings (Figure 5). It appeared that all normalized PGE_2 values, regardless of treatment, decreased from d 0 to d 3. PGE_2 production numerically increased in horses fed either CO or SBO diets between d 10 and d 17, though it was not

significantly different ($P > 0.05$). Horses consuming MCO diets had declining PGE_2 concentrations by d 17; however, there were no statistical variation between treatments ($P > 0.05$).

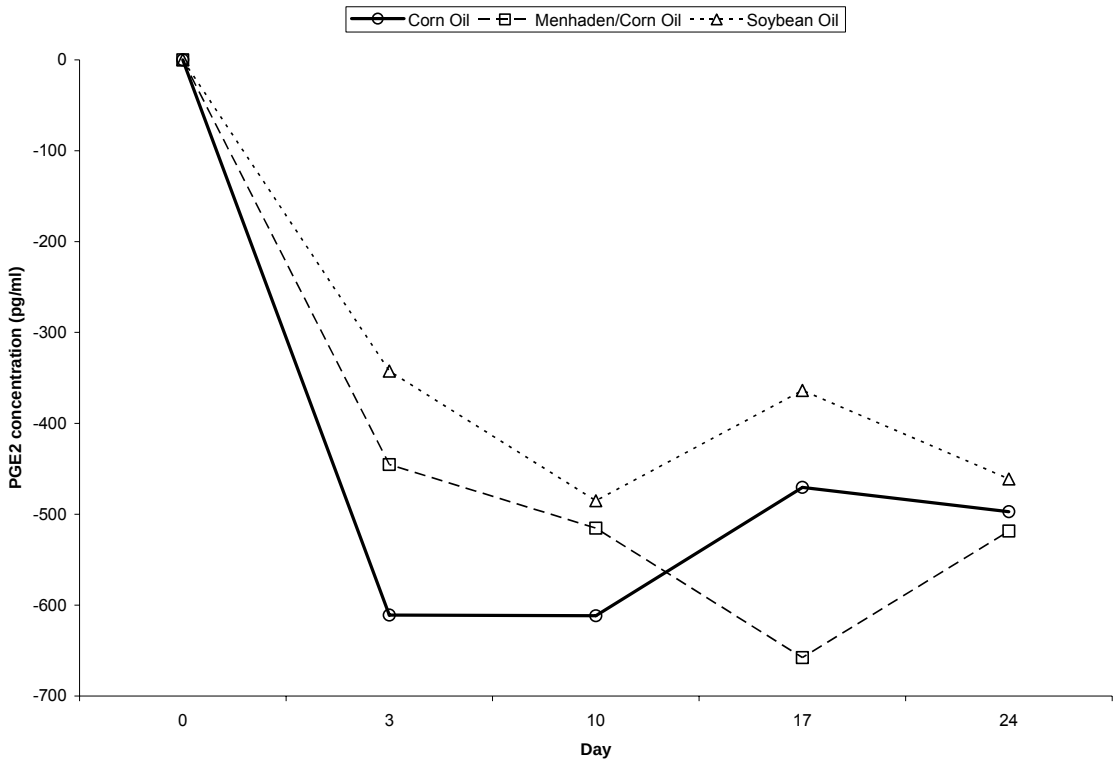

Figure 5. Normalized mean PGE_2 concentration over a 24 day period

As similar to Osteocalcin data, there were significant period effects in mean plasma PGE_2 concentration ($P<0.05$) and in normalized PGE_2 ($P<0.05$) (Table 13). There were numerical decreases in PGE_2 concentrations from baseline values through the end of the trial (Figure 6). It appears that there was an adaptation to the exercise either physiologically or metabolically in the yearlings.

Table 13. PGE_2 concentration (all groups)

Period	1	2	3	Mean
Mean PGE_2 (ng/ml)	1150.29[a]	890.11[a,b]	770.98[b]	931.26
SEM	119.88	72.56	51.93	50.12
Normalized PGE_2 (ng/ml)	-248.56[c]	-482.79[c,d]	-636.96[d]	-460.94
SEM	99.01	96.33	118.18	61.92

[a,b]Row means not sharing superscripts differ (P = 0.012)
[c,d]Row means not sharing superscripts differ (P = 0.046)

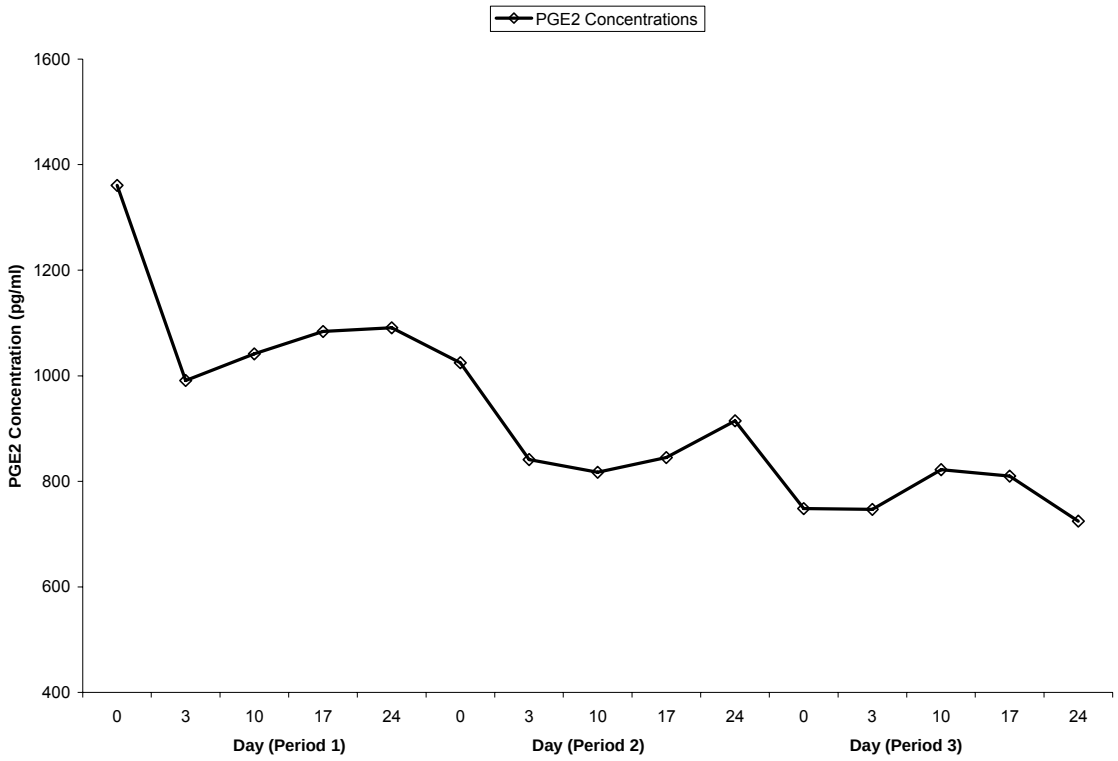

Figure 6. Mean PGE_2 for all groups throughout the trial

Fibrinogen

Plasma samples collected on days 0, 3, 7, 10, 14, 17, 21, 24 and 28 of each period were analyzed for fibrinogen concentration. Mean fibrinogen is shown in Appendix 7. A significant treatment effect on mean fibrinogen concentration was not observed

(P = 0.886) Mean fibrinogen separated by treatments and by day of period are illustrated in Figure 7.

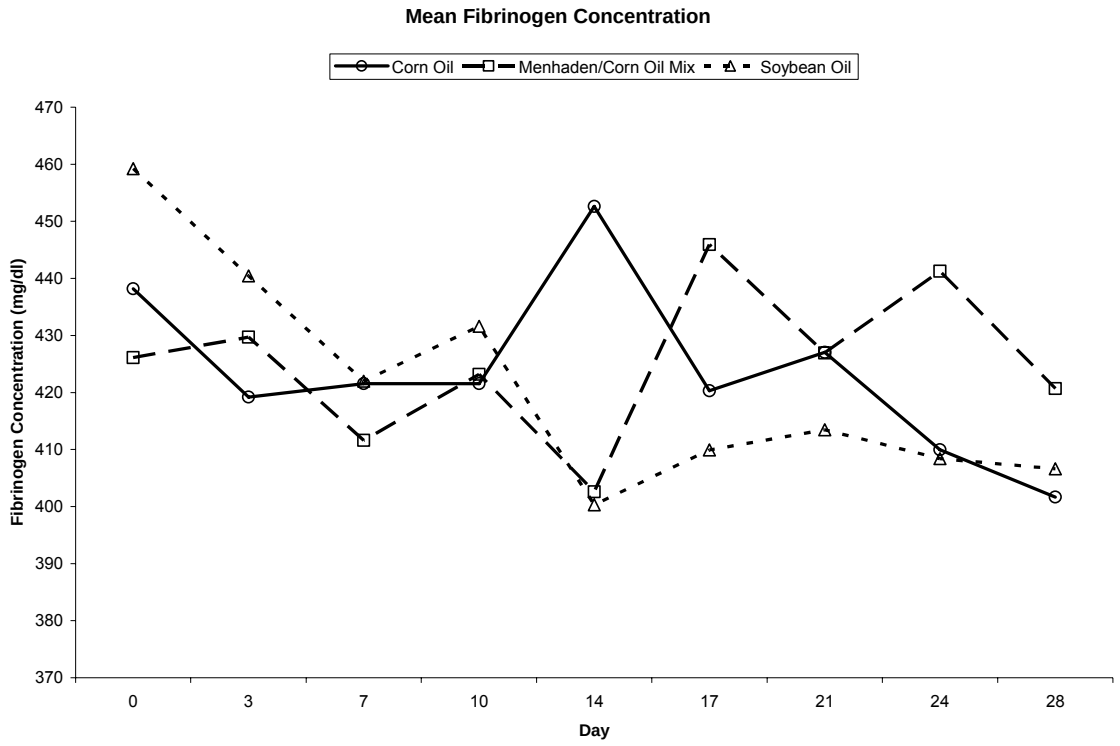

Figure 7. Mean fibrinogen concentration by diet over a 28 day period

There was a significant period effect with mean fibrinogen (P < 0.001) (Table 14). Overall mean fibrinogen values were highest during period 1 and subsequently decreased throughout period 2 with lowest mean concentrations occurring within period 3 (Figure 8). This pattern is similar to the PGE_2 measurements previously mentioned.

Table 14. Mean fibrinogen concentrations among all groups

Period	1	2	3
(mg/dl)	460.79[a]	415.04[b]	401.16[c]
SEM	5.80	2.75	1.63

[a,b,c] Row means not sharing superscripts differ ($P < 0.001$)

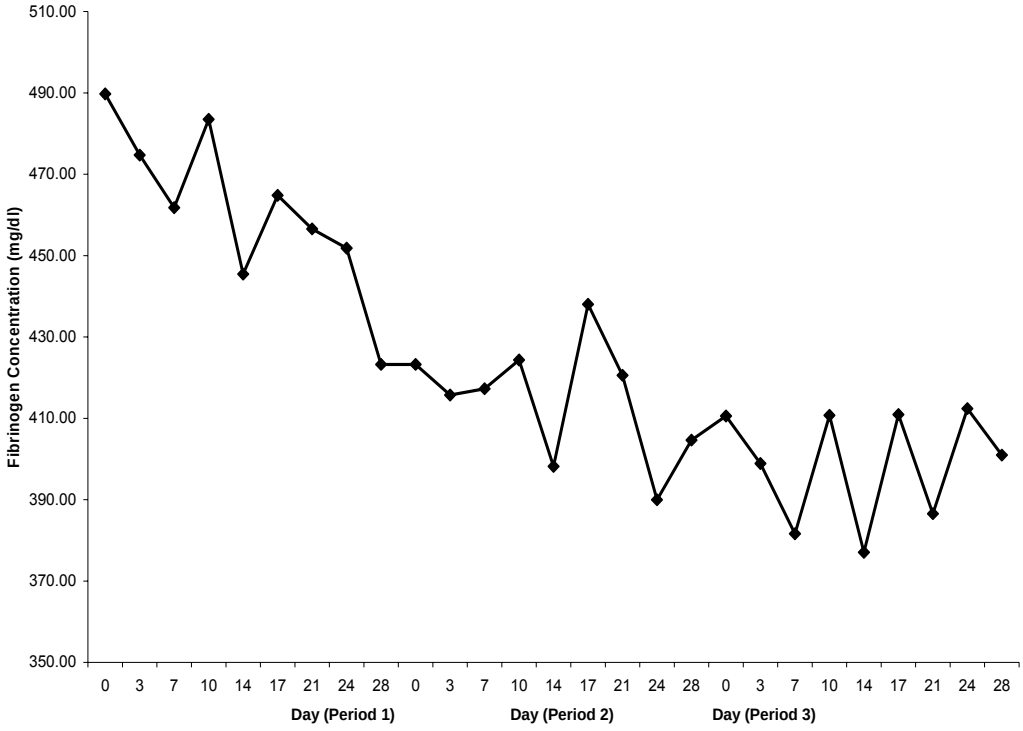

Figure 8. Mean fibrinogen concentrations for all groups throughout trial

Despite the randomization of yearlings into treatment groups, there appeared to be a pattern in the group order of mean fibrinogen values (Figures 9-11). Overall Group B were numerically higher during periods 1 and 2 than Group C, while Group C was numerically higher than Group A during the same periods. A numerical change in the observed group order appears during period 3 (Figure 11). Due to the apparent pattern,

data were normalized for each horse back to d 0 of each period to evaluate change in fibrinogen concentrations over 28 days.

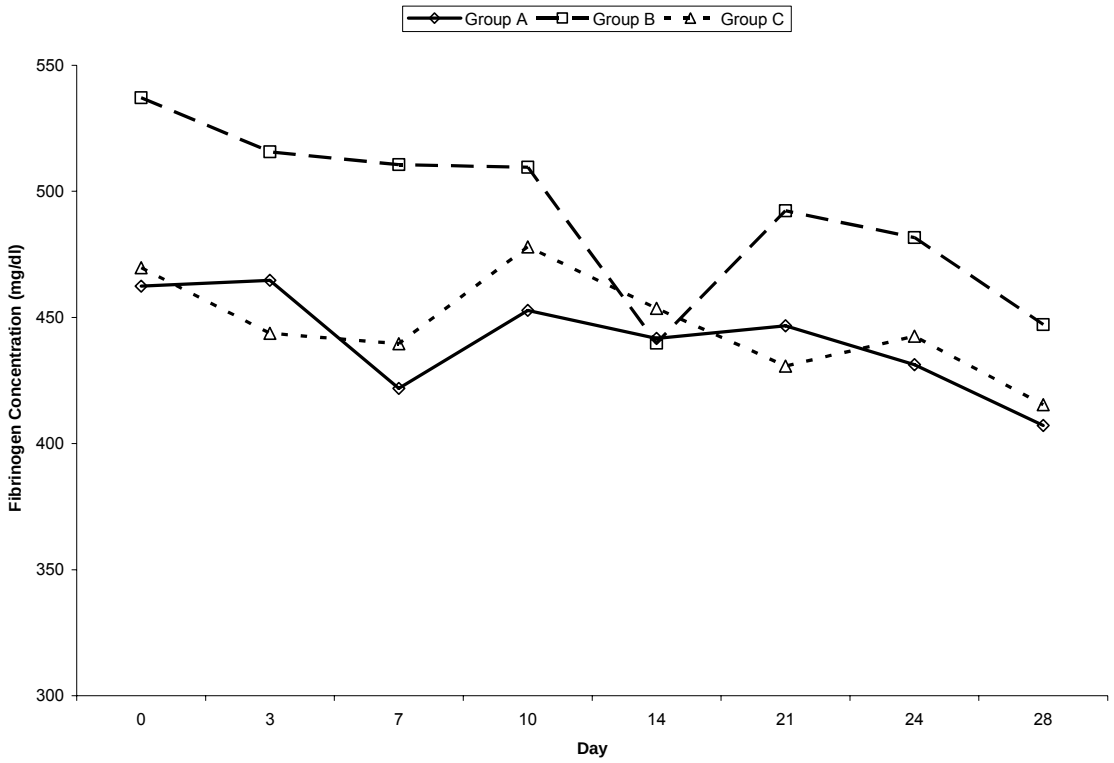

Figure 9. Mean fibrinogen concentrations by group: Period 1

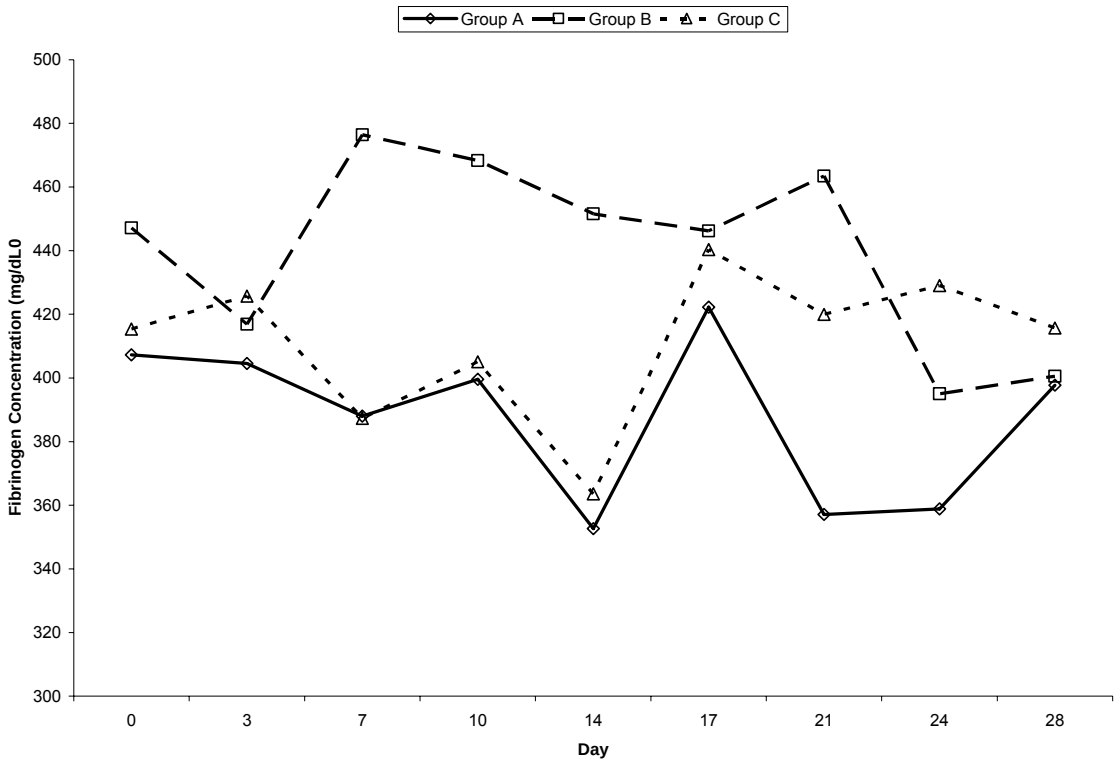

Figure 10. Mean fibrinogen concentrations by group: Period 2

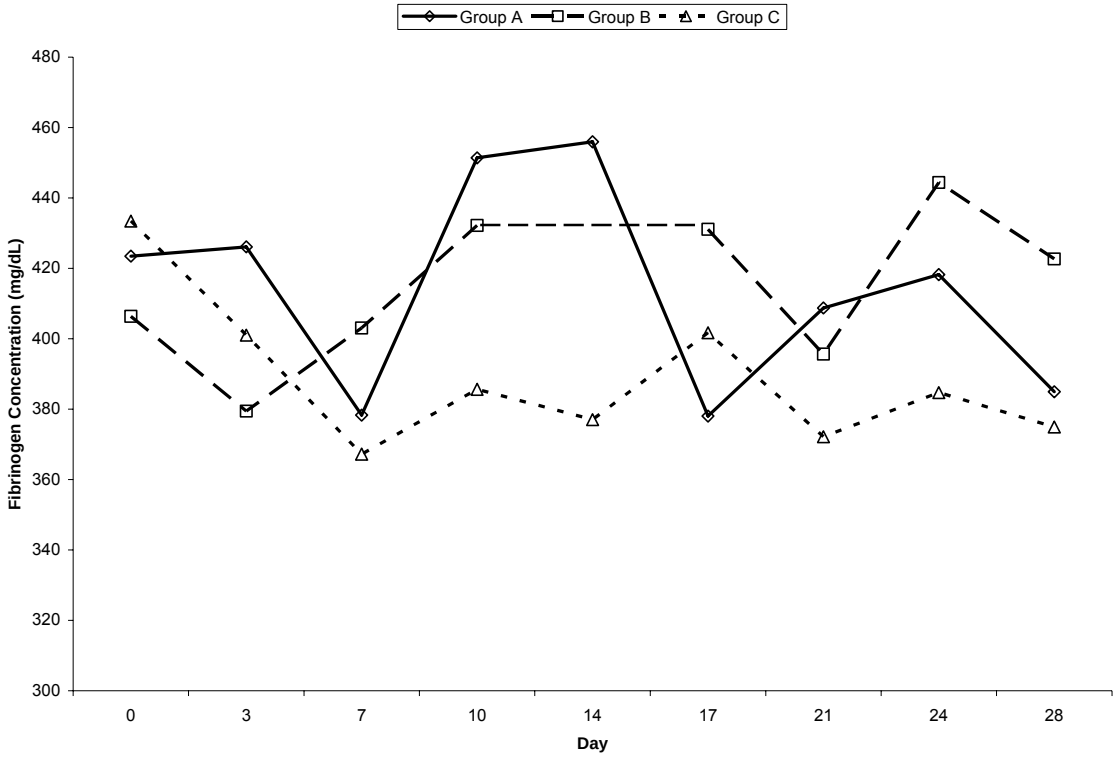

Figure 11. Mean fibrinogen concentrations by group: Period 3

Mean normalized data for fibrinogen concentrations is shown in Appendix 8 and illustrated in Figure 12. When normalized data were analyzed, there was a significant effect of diet on fibrinogen concentrations ($P < 0.001$). Average normalized fibrinogen measurements among treatments are summarized in Table 15.

Table 15. Normalized fibrinogen concentrations by treatment

Treatment	CO	MCO	SBO
Mean Fibrinogen concentration (mg/dl)	-16.03[a]	4.31[b]	-37.73[c]
SEM	4.37	5.49	4.91

[a,b,c] Row means not sharing superscripts differ ($P < 0.001$)

Mean normalized fibrinogen concentrations were lowest in horses fed SBO diet ($P < 0.007$) and highest for horses fed MCO diet ($P < 0.014$). Horses consuming CO diet expressed fibrinogen concentrations intermediate to measurements recorded from horses fed either SBO or MCO diets ($P < 0.014$).

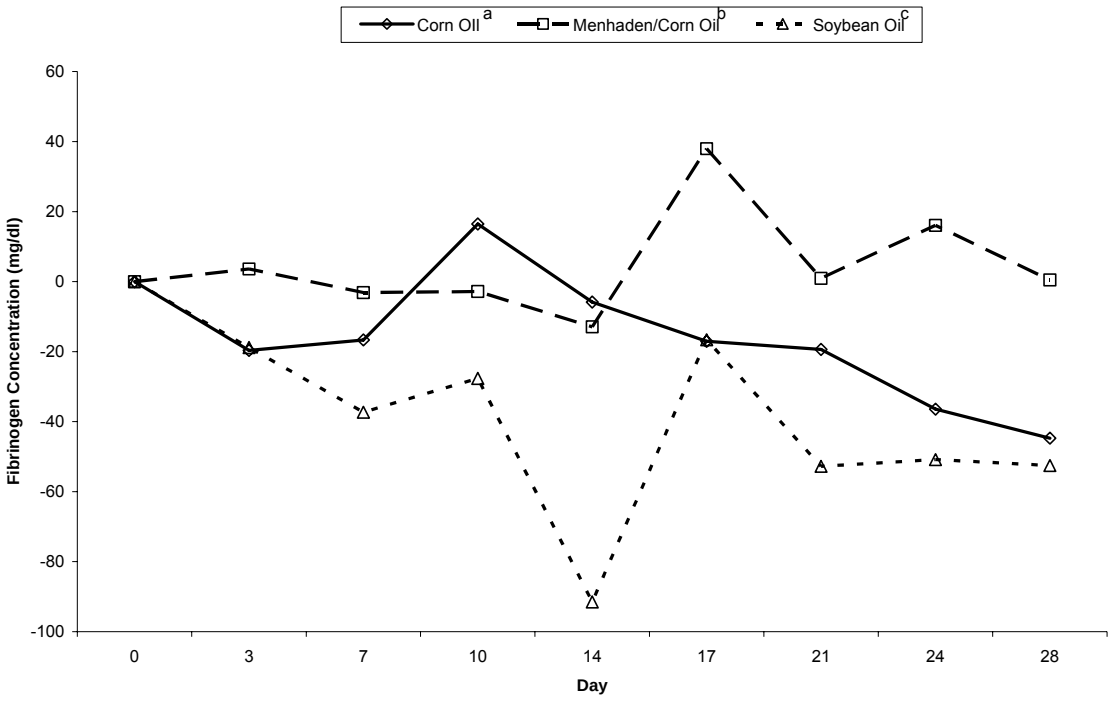

Figure 12 Normalized fibrinogen concentrations by diet over a 28 day period.
[a,b,c] Diets lacking common superscripts differ ($P < 0.001$)

CHAPTER V

GENERAL DISCUSSION

Experimental Design

The experimental model designated for the present study was a triplicated 3 X 3 Latin Square designed to evaluate the effect of dietary PUFAs on bone biochemical markers and inflammatory response in exercising yearlings. This model is ideal when there are a limited number of animals to use. A switchback design creates a larger number of observations with higher degrees of freedom, resulting in a stronger statistical output if every period can be replicated in an identical manner. However, when the Latin Square was applied in this study, there was a significant effect of period. It is difficult to interpret if the dietary effects on the variables tested are a result of the actual diet or if it is confounded by the effect of period.

A recent study investigating PUFA supplementation on inflammation in exercising horses used the same experimental model and had no period effects (Wilson et al, 2003). In the previous study, horses had been engaged in extensive training programs at their respective facilities, and upon arriving at the research center, underwent a 21-d pre-trial conditioning period to achieve an equal physical state among all horses. The exercise protocol remained the same throughout the trial with no increase in intensities. A standardized exercise test (SET) was conducted on d 29, subjecting all horses to an exhaustive level of exercise, with inflammation markers measured prior to the SET and for 7 days after. The use of a SET established a more controlled observation of the variables in question and confirmed results previously published that indicate fibrinogen concentrations immediately drop after acute phase response to inflammation and then

subsequently increase in the days following (Allen and Kold,1988). The use of a SET in the present study would have been beneficial in properly evaluating diet effect on inflammation markers. Exercise protocol for the present study was constant throughout as well, however it is likely the 14 d background period was not sufficient in establishing an equal conditioned state among the yearling horses. In Wilson's study (2003), horses were older; 3 and 4 yr of age, while the horses in the present study averaged 14 mo of age at the beginning of the trial. It is possible that there are age differences in sub-clinical inflammation thresholds among horses.

Fatty Acid Profiles

The dietary consumption of various oil sources incorporated at 8% of the diet, resulted in changes in plasma concentration (% by weight) of identifiable fatty acids from basal measurements in the horse. Percent change in fatty acid concentration of C14:0 (mystric acid), C16:0 (palmitic acid), C18:0 (steric acid), C18:1 (oleic acid), C18:2 (linoleic acid) and C18:3 (alpha linoleic acid) on d 28 did not differ among diets. These results are consistent with other studies evaluating fatty acid profiles in horses fed various oil sources (Wilson et al., 2003). Horses fed SBO diets had a greater percent decrease in C16:1 (palmoleic acid) concentration when compared to CO fed horses, however the decrease was similar to C16:1 concentration decreases found in horses fed MCO.

Arachidonic acid concentrations were significantly higher at d 28 in horses consuming MCO compared to horses fed the CO. These results are similar with findings of Hall and associates (2004), where horses fed a menhaden oil diet for 12 weeks had higher concentrations of AA than horses supplemented with corn oil over the same timeframe; concluding that the higher amounts of AA found in the MCO diet are a result

of the exogenous amount of AA in the menhaden oil. Despite an increase in Linoleic Acid; a precursor for Arachidonic Acid, there was a decrease in Arachidonic Acid concentrations (% by weight) in horses fed the CO diet for 28 days, indicating that perhaps an inhibition of LA elongating to AA is occurring. However, it should be stressed that fatty acid profiles in this study are expressed in percentage by weight units meaning that as one fatty acid changes in percentage by weight; it results in the percent change of another fatty acid. The interpretation of the fatty acids in this manner is a result of how the plasma samples were assayed.

In horses consuming a menhaden/corn oil supplemented-diet, C20:5 (Eicosapentenoic Acid) and C22:6 (Docosahexenoic Acid) increased 3 fold and nearly 7 fold over baseline values, respectively. Percent changes in both EPA and DHA profiles in MCO fed horses were significantly higher compared to SBO fed horses. Changes in DHA profiles were different between MCO horses and CO horses with MCO horses having significantly higher increases at d 28. Though numerically different, there was no statistical difference in percent change of EPA among MCO and CO fed horses. In corn oil fed horses, there was an observed increase in C18:3 (alpha linolenic acid), the parent fatty acid to EPA. Therefore it is within reason to assume that the increase of EPA in these horses is a result of the increase in C18:3. The incorporation of C20:5 and C22:6 in the equine blood observed in the present study is consistent with previous research utilizing menhaden oil, or fish-derived EPA and DHA supplements as an omega 3 source in the diet of the mature horse (O'Conner et al., 2001, King et al., 2005) and in the yearling horse (Vineyard et al., 2006).

Supplementation with mechanically extracted soybean oil resulted in decreases in all identified fatty acids with the exception of C 18:0 (steric acid), C18:2 (linoleic acid) and C22:6 (docosahexenoic acid). Numerically, C18:2 profiles in SBO fed horses were of the highest profiles and percent change from baseline measurements; however, there were no significant differences in linoleic acid profiles between the diets.

Fatty acid results reported here are consistent with other literature indicating blood fatty acid profiles are directly associated with the dietary intake of those fatty acids (Bjerve et al., 1993).

Bone Biochemical Markers

There was no effect of diet or day on Osteocalcin concentration or ICTP concentrations in horses fed various ratios of omega 6: omega 3 PUFAs. These results are consistent with a previous study where Osteocalcin concentrations were unaltered in young growing rats fed an elevated omega-3 diet (Kelly et al., 2003). The observed pattern in normalized mean OC concentrations was similar to a previous study evaluating Osteocalcin levels in yearlings undergoing various forms of exercise (Fenton et al., 1999).

Due to the observed period effect on Osteocalcin measurements in the present study, data can not confirm or deny that consistency of these results. The experimental design used in this study is not appropriate for evaluating PUFA influence on bone activity in rapidly, growing yearlings, as the timeframe of 28 d appears to be insufficient in identifying significant changes to sensitive bone markers. Results suggest that the exercise protocol used was insufficient in stimulating a bone degradation response in ICTP or a bone turnover response for Osteocalcin.

This was the first study to investigate polyunsaturated fatty acid effects on bone activity in the horse. Future research is needed to further identify omega 6 and omega 3 responsibilities in equine skeletal health.

Inflammation Markers

There was no significant difference in PGE_2 measurements as a result of diet. There was a significant period effect, with adaptation to exercise most likely being the contributing factor. PGE_2 concentrations among all horses were extremely variable, with high standard deviations resulting. The assay used in this study had been previously validated using equine synovial fluid with little variation among measurements (Munsterman et al., 2005). The variability among horses may be explained by plasma sample processing methods. Previous literature has indicated the addition of a prostaglandin inhibitor to samples prior to storage (Bertone et al., 2001). The decision to use PGE_2 as an inflammation marker was determined after the samples had been processed and stored; therefore an inhibitor was not added. However, PGE_2 concentrations defined in this study were within the kit standards range, therefore the measurements are reliable data points. There is extensive information available on PGE2 concentrations in equine synovial fluid, exudates and cultured medias, however there is little to no information regarding PGE_2 levels in resting or exercising horses. One study reported mean plasma concentrations of PGE_2 in normal horses to be 187.18 pg/ml (Owens et al.,1995). These measurements are significantly lower than concentrations observed in the present study, however, horses in Owens et al., study were measured at rest and then directly after light hand exercise. PGE_2 concentrations do not instantly increase after a stimulus; therefore samples taken that soon after cessation of exercise are

not reliable indicator of an inflammatory response. Past research indicated exudates PGE_2 increased 2 hrs after an inflammatory induction with levels peaking at 12 hrs (Higgins and Lee, 1984a) and remaining elevated up to 72 hrs (Guthrie et al., 1996). Sample collection in this study was taken approximately 18 hrs post exercise; therefore concentrations may be of a recovery dilution rather than of peak levels. This is one of the first studies to investigate PGE_2 in horse plasma under exercising conditions. Further investigations are necessary to identify measurements of PGE_2 over a 24 hr period in both resting and exercising horses to provide a template of normal plasma levels as well as establish if diurnal variation exists in plasma PGE_2, as it does in other blood-borne biological markers.

Fibrinogen was the second marker used to evaluate inflammation. There was a significant difference in normalized fibrinogen. However, there was a significant period effect with this set of data, complicating the reliability of the results. Horses fed SBO had a significantly lower normalized mean concentration of fibrinogen when compared to horses consuming either CO or MCO diets. These results are consistent with previous research where horses consuming mechanically extracted soybean oil had lower fibrinogen concentrations compared to horse fed corn oil (Wilson et al., 2003). When horses in the present study consumed CO, the mean fibrinogen concentration was significantly lower ($P<0.001$) than horse fed MCO diet. These results are inconsistent with findings in other mammals fed menhaden oil as a mediator of inflammation (Calder 2002). Menhaden oil in this study however was mixed with corn oil, possibility resulting in a competitive inhibition between omega 6 derivatives and omega 3 derivatives, allowing fibrinogen levels to remain elevated.

Other research using equine models to measure inflammation marker production concluded that elevated n3 diets resulted in the decreased production of PGE_2 levels in brochoalevolar lavage fluid cells (Hall et al., 2004) and in synovial membrane explant cultures (Munsterman et al., 2005). It is possible that the level of EPA and DHA supplied in the diet was not sufficient to alter biological responses. However, Caughey et al., (1996), reported that EPA concentration of approximately 1% of total mononuclear cell membrane fatty acid decreased cytokine production and that any increase in % EPA did not result in additional decrease of cytokines. The same study concluded that when 1.6g of EPA was supplemented, 1.6% of EPA was incorporated into the mononuclear cell membrane. Though plasma concentrations of fatty acids can not be compared to cell membrane concentrations, the mean percent by weight of EPA and DHA in the horses involved in this study was 1.36 % and 1.64% respectively. The evaluation of inflammation markers and their assuage as a result of the diet is a relatively new topic in equine research and needs further investigation.

CHAPTER VI

SUMMARY AND CONCLUSIONS

In recent years, there has been an increased interest in dietary fat, and its formulation in the diet has been reevaluated. The dramatic change in food formulations has led to a greater dietary intake of plant oils such as corn, safflower, and soybean oil. These oils are high in linoleic acid, and have resulted in an elevated ratio of ω-6: ω-3 fatty acids in human diets during the 20th century (Watkins et al., 2001b). However, recent studies have indicated that a high consumption of ω-6 PUFAs in conjunction with a low consumption of ω-3 PUFAs may contribute to the development of some chronic diseases, cancer, cardiovascular disease and skeletal disease (Watkins et al., 2003). Based on these discoveries, nutritional research has turned a large focus in the direction of ω-3 PUFAs and their role in the diet. Evaluation of the omega 6 and omega 3 fatty acids within the equine diet is a relatively new subject, with little data to confirm if the benefits seen in other species can be duplicated in the horse.

Nine quarter horse yearlings were blocked by age and gender and randomly assigned one of three diets within a Latin Square experimental model. Diets were supplemented with a variety of oil to achieve different levels of omega 6: omega 3 polyunsaturated fatty acid ratios. Corn oil supplementation (CO) resulted in a 21.05:1 omega 6: omega 3 PUFA ratio; Menhaden/Corn oil mix (MCO) resulted in a 12.7:1 ratio and the mechanically extruded Soybean Oil diet (SBO) had a calculated omega 6: omega 3 ratio of 15.6:1.

After a 14 day exercise pre-conditioning period, horses were placed in a 28 day diet and experimental exercise adaptation period. Blood samples were harvested bi-weekly over the course of each period for evaluation of bone activity and inflammation response to diet, as well as to determine plasma fatty acid profiles in horses fed varying ratios of omega 6: omega 3 PUFAs.

The bone formation marker, Osteocalcin, was not different among the diets, nor was the bone degradation marker ICTP. The inflammation markers PGE_2 and fibrinogen did not change as a result of diet. Fibrinogen data normalized from day 0 of each period indicate a significant difference between diets; with SBO fed horses exhibiting the lowest concentrations of fibrinogen when compared to CO fed horses and MCO fed horses ($P< 0.001$). Data from the bone markers suggests that exercise was not intense enough to induce bone degradation or formation in the yearlings. Inflammation markers were recorded at measurements consistent with previous research to be sub-clinical levels of inflammation; however, these values were only seen in the first period with marked decreases in mean concentrations occurring in the preceding periods regardless of diet. A strong period effect was evident with data collected from both inflammation markers as well as for the bone turnover marker Osteocalcin. An adaptation to the exercise protocol and an unforeseen horse effect are likely the contributing factors to the perceived effect.

The increased incorporation of the long chain fatty acids into equine plasma, arachidonic acid, eicosapenteonic acid and docosahexonic acid in horses fed the MCO diet compared to the other diets is consistent with previous research. Concentrations of C18:2 (Linoleic Acid), the parent metabolite to arachidonic acid, were not significantly different between diets. Despite the menhaden oil being mixed with corn oil, there

appears to be sufficient amounts of EPA and DHA being measured in the plasma; mean increases were 4 fold and 6 fold above baseline values, respectively. This is one of the first studies investigating the short term (28 d) incorporation of EPA and DHA in horses. There was no significant carry over effect of these essential fatty acids found in equine plasma which is consistent with previous findings where concentrations of EPA and DHA were returning to basal levels as soon as d 9 after cessation of omega 3 supplementation (King et al., 2005).

The data presented indicated the experimental model used was insufficient in properly evaluating the multiple variables under question. The use of a switchback model prevented an accurate assessment of dietary treatment on biochemical bone markers in rapidly growing yearlings. The physiological change occurring in bone of this age group makes it difficult to measure a precise change as a result of the diet with this type of experimental design. A substitute method could be a straight blocking of the young horses by gender and age, randomly assigning each group one of the three diets, and having no changing of treatments.

Incorporating a standard exercise test at the end of each period in the present model would have provided a consistent measurement among horses regarding treatment influence to the inflammatory response. Subjecting the horses to an exhaustive workout, then measuring inflammatory reaction and recovery over a certain period of time, would offer a more confident evaluation of sub-clinical inflammatory response to treatment in exercising yearlings. A standard exercise test for the evaluation of acute phase protein concentrations has been successfully used in previous studies (Wilson et al., 2003, Wagner et al., 2006, Texas A&M University; unpublished results).

Due to an apparent period effect, the hypothesis of altering omega 6: omega 3 ratios in the equine diet may influence inflammatory response and bone activity in the young exercising horse, can not be confidently confirmed nor denied. Despite the apparent effect of period on the regarded variables, the patterns seen within the collected data quantify a need for further investigations in this area of equine nutrition. Future queries would benefit from the evaluation of PUFA regulation of eicosanoids in bone metabolism in the young horse as well as the mediating properties elevated omega 3 diets have in the inflammatory response. It would be useful to determine if dietary PUFA do in fact influence calcium absorption in horses, as seen in other species. Previous research investigating fat-supplemented diets in equines determined that dietary fat had no bearing on the regulation of calcium (Bowman, 1977).

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APPENDICES

APPENDIX 1. MEAN FATTY ACID COMPOSITION (% BY WEIGHT) ALL HORSES, DAY 0

Fatty Acid	All Groups	
	Mean	SEM
C14:0	0.11	0.02
C16:0	9.92	0.61
C16:1(n-7)	0.29	0.01
C18:0	17.86	0.35
C18:1(n9)	10.31	0.44
C18:2(n6)	52.91	0.45
C18:3(n3)	1.21	0.36
C20:4(n6)	1.93	0.06
C20:5(n3)	0.02	0.01
C22:6(n3)	0.10	0.03

APPENDIX 2. MEAN ICTP CONCENTRATION (ng/ml) BY DIET AND DAY

Day		0	14	28
Mean ITCP Concentration (ng/ml)	CO	27.41	21.04	24.08
	SEM	4.25	1.34	3.54
	MCO	22.43	20.41	21.16
	SEM	2.53	1.31	2.08
	SBO	25.33	23.53	20.26
	SEM	6.00	2.72	1.53
	All Groups	25.06	21.66	21.83
	SEM	2.36	1.09	1.44

APPENDIX 3. NORMALIZED MEAN ITCP CONCENTRATION (ng/ml) BY DIET AND DAY

Day		0	14	28
ITCP Concentration (ng/ml)	CO	0.00	-1.96	1.08
	SEM	0.00	1.47	2.48
	MCO	0.00	-4.57	-3.81
	SEM	0.00	2.73	3.74
	SBO	0.00	0.01	-3.27
	SEM	0.00	1.07	1.61
	All Groups	0.00	-2.18	-2.00
	SEM	0.00	1.11	1.59

APPENDIX 4. MEAN OSTEOCALCIN CONCENTRATION BY DIET AND DAY

Day		0	14	28
Osteocalcin concentration (ng/ml)	CO	66.65	55.41	61.79
	SEM	8.91	5.45	5.40
	MCO	53.03	56.96	62.26
	SEM	5.47	5.05	7.02
	SBO	55.56	57.52	64.42
	SEM	4.77	5.45	6.07
	All Groups	58.41	56.63	62.82
	SEM	3.92	2.96	3.45

APPENDIX 5. NORMALIZED MEAN OSTEOCALCIN CONCENTRATIONS (ng/ml) BY DIET AND DAY

Day		0	14	28
Osteocalcin concentration (ng/ml)	CO	0	-9.22	-2.84
	SEM	0	6.07	4.63
	MCO	0	-2.13	3.16
	SEM	0	4.40	6.28
	SBO	0	-7.89	-0.99
	SEM	0	4.11	5.38
	All Groups	0	-6.41	-0.22
	SEM	0	2.81	3.07

APPENDIX 6. MEAN FIBRINOGEN CONCENTRATIONS BY DIET AND DAY

		CO	MCO	SBO	All Groups
Day					
(mg/dl)	*0*	438.203	426.118	459.251	441.191
SEM		17.4263	17.2495	22.932	11.0819
	3	419.183	429.716	440.431	429.777
		23.8941	16.4935	21.5694	11.7077
	7	421.543	411.606	421.96	418.63
		21.6949	12.6699	24.6601	11.5847
	10	421.543	423.201	431.61	438.915
		19.6633	14.224	21.4637	11.0445
	14	452.6	402.61	400.32	420.498
		18.25	24.3005	18.409	12.4417
	17	420.286	445.935	409.948	425.964
		20.9233	15.1282	20.7775	11.1126
	21	427.019	426.919	413.45	422.641
		19.2097	13.8866	28.8568	11.7029
	24	409.963	441.246	408.408	419.414
		11.8131	14.5722	20.5041	9.62587
	28	401.663	420.68	406.61	409.958
		16.7923	9.91258	21.2436	9.36807
Mean (mg/dl)		427.499	426.077	422.927	
SEM		6.48635	4.91481	7.56442	

APPENDIX 7. NORMALIZED MEAN FIBRINOGEN CONCENTRATIONS BY DIET AND DAY

		CO	MCO	SBO	All Groups
Day					
(mg/dl)	*0*	0	0	0	0
SEM		0	0	0	0
	3	-19.68	3.59	-18.82	-11.63
		14.73	9.91	12.18	7.57
	7	-16.66	-3.14	-37.29	-19.64
		13.8	18.35	15.93	9.27
	10	16.45	-2.83	-27.64	-5.59
		10.57	14.11	11.84	7.73
	14	-5.84	-12.94	-91.52	-39.74
		10.22	17.2	10.85	12.26
	17	-17.04	37.97	-16.59	1.42
		9.95	10.94	18.57	9.33
	21	-19.37	0.93	-52.77	-23.91
		14.03	18.89	13.89	9.91
	24	-36.43	16.06	-50.84	-24.82
		13.25	24.24	7.24	10.58
	28	-44.73	0.47	-52.64	-31.82
		15.03	21.36	15.54	10.92
Mean (mg/dl)		-16.03	4.31	-37.73	
SEM		4.38	5.49	4.91	

APPENDIX 8. TOTAL DIET N6:N3 POLYUNSATURATED FATTY ACID RATIOS

n6:n3 Ratios		C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:4	C20:5	C22:6
CO diet	grain	0.0676	8.1692	0.0832	1.4404	16.9988	20.358	0.9308	0.1092	0.1404	0.0364
	hay	0.408	9.276	0.052	1.372	1.876	5.924	8.152	0.808	0.156	0.332
	oil	0.0408	0.948	0.0352	0.1792	2.1424	4.468	0.0784	0	0	0
Total		0.5164	18.3932	0.1704	2.9916	21.0172	30.75	9.1612	0.9172	0.2964	0.3684
n6:n3 ratio	3.22										
MCO diet	grain	0.0676	8.1692	0.0832	1.4404	16.9988	20.358	0.9308	0.1092	0.1404	0.0364
	hay	0.408	9.276	0.052	1.372	1.876	5.924	8.152	0.808	0.156	0.332
	oil	0.1456	1.028	0.1992	0.1944	1.8152	3.4416	0.0936	0.0128	0.2472	0.2704
Total		0.6212	18.4732	0.3344	3.0068	20.69	29.7236	9.1764	0.93	0.5436	0.6388
n6:n3 ratio	2.95										
SBO diet	grain	0.0676	8.1692	0.0832	1.4404	16.9988	20.358	0.9308	0.1092	0.1404	0.0364
	hay	0.408	9.276	0.052	1.372	1.876	5.924	8.152	0.808	0.156	0.332
	oil	0.0056	0.8496	0.0056	0.3336	1.6624	4.4072	0.6464	0	0	0
Total		0.4812	18.2948	0.1408	3.146	20.5372	30.6892	9.7292	0.9172	0.2964	0.3684
n6:n3 ratio	3.04										

APPENDIX 9. DAY 0 PLASMA FATTY ACID COMPOSITON (% BY WEIGHT)

Horse	Diet	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:4	C20:5	C22:6
1A		0.06955	12.3215	0.29655	17.94015	8.97495	53.25195	2.61375	2.00025	0	0
2A		0.0721	9.5319	0.2638	17.6457	9.672	54.1374	1.8966	1.8938	0	0.0709
3A		0.12955	5.9978	0.36715	15.67305	11.6734	53.4635	2.72075	1.6973	0	0
1B		0.0552	8.9186	0.2287	17.6281	10.9786	54.9316	0.2801	2.0117	0	0
2B		0.0808	10.4323	0.30955	18.2171	12.7907	51.6306	0.1481	2.1726	0	0.1102
3B		0.1139	10.0947	0.2651	18.8069	9.5635	51.3021	0.2369	2.1829	0	0.1766
1C		0.2175	9.4894	0.3358	19.5641	9.3915	52.5964	0.9105	1.6588	0.0857	0.1851
2C		0.1617	11.7043	0.3415	17.6951	8.971	53.7895	1.9832	1.8582	0	0.2332
3C		0.0967	10.8639	0.2777	17.5886	10.7863	51.1205	0.1569	1.9165	0.0728	0.1202

APPENDIX 10. MEAN PGE_2 CONCENTRATIONS (pg/ml) BY DIET AND DAY

	PGE_2 (pg/ml)				
	D0	D3	D10	D17	D24
CO	1028.556	749.7105	842.5743	890.2074	863.4798
SEM	192.6991	135.9845	184.7216	148.566	185.4606
MCO	928.1476	915.2614	943.837	721.5244	983.6442
SEM	160.1556	160.4418	251.7125	109.8118	186.8593
SBO	1176.841	926.7619	875.4658	1121.421	1011.877
SEM	319.327	236.1728	211.6033	253.7075	195.7115
All Groups	1044.515	858.8832	884.4436	910.2173	947.9935
SEM	131.5259	97.43019	119.1397	104.198	105.9273

APPENDIX 11. ICTP AND OSTEOCALCIN CONCENTRATIONS

Horse	Diet	Day	ICTP (ng/ml)	Osteocalcin (ng/ml)
1A	CO	0	34.29	69.9353
1A	CO	14	27.52	74.7396
1A	CO	28	50	72.15
1A	MCO	0	50	72.15
1A	MCO	14	27.062	64.187
1A	MCO	28	23.288	74.0613
1A	SBO	0	23.288	74.0613
1A	SBO	14	21.317	64.76
1A	SBO	28	23.886	52.426
2A	CO	0	28.27	80.178
2A	CO	14	20.547	71.2465
2A	CO	28	23.152	77.161
2A	MCO	0	23.152	77.161
2A	MCO	14	18.703	79.069
2A	MCO	28	20.01	88.656
2A	SBO	0	20.01	88.656
2A	SBO	14	16.455	60.30375
2A	SBO	28	18.434	87.89
3A	CO	0	19.651	49.8305
3A	CO	14	20.537	52.827
3A	CO	28	16.922	57.6035
3A	MCO	0	16.922	57.6035
3A	MCO	14	15.76	60.684
3A	MCO	28	16.626	64.326
3A	SBO	0	16.626	64.326
3A	SBO	14	15.376	64.78975
3A	SBO	28	15.233	57.771
1B	MCO	0	24.785	51.8866
1B	MCO	14	19.082	64.045
1B	MCO	28	16.418	42.8946
1B	SBO	0	16.418	42.8946
1B	SBO	14	21.37	45.05433
1B	SBO	28	18.833	67.379
1B	CO	0	18.833	67.379
1B	CO	14	17.201	61.355
1B	CO	28	17.886	53.3
2B	MCO	0	17.37	44.17
2B	MCO	14	25.157	62.95575
2B	MCO	28	33.635	92.143
2B	SBO	0	33.635	92.143
2B	SBO	14	38.515	82.427
2B	SBO	28	29.864	82.441
2B	CO	0	29.864	82.441
2B	CO	14	22.306	72.4345
2B	CO	28	20.016	84.515
3B	MCO	0	25.125	63.024
3B	MCO	14	20.246	61.53175

APPENDIX 11 (CONT)

Horse	Diet	Day	ICTP(ng/ml)	Osteocalcin(ng/ml)
3B	MCO	28	25.811	59.91075
3B	SBO	0	25.811	59.91075
3B	SBO	14	27.043	63.3525
3B	SBO	28	19.127	55.732
3B	CO	0	19.127	55.732
3B	CO	14	22.68	45.779
3B	CO	28	21.367	45.538
1C	SBO	0	15.255	46.045
1C	SBO	14	16.086	31.254
1C	SBO	28	15.687	32.878
1C	CO	0	15.687	32.878
1C	CO	14	15.594	33.726
1C	CO	28	17.575	35.723
1C	MCO	0	17.575	35.723
1C	MCO	14	17.219	30.918
1C	MCO	28	12.771	33.071
2C	SBO	0	36.001	60.917
2C	SBO	14	34.016	68.3926
2C	SBO	28	22.804	57.3435
2C	CO	0	22.804	57.3435
2C	CO	14	25.88	56.0905
2C	CO	28	31.025	74.6135
2C	MCO	0	31.025	74.6135
2C	MCO	14	23.399	54.504
2C	MCO	28	23.577	66.7083
3C	SBO	0	24.748	59.725
3C	SBO	14	21.619	37.335
3C	SBO	28	18.49	85.9285
3C	SBO	0	18.49	85.9285
3C	CO	14	17.094	30.51
3C	CO	28	18.794	55.503
3C	MCO	0	18.794	55.503
3C	MCO	14	17.033	34.783
3C	MCO	28	18.278	38.531

APPENDIX 12. FIBRINOGEN CONCENTRATION (mg/dl)

Horse	Diet	Day	Fibrinogen (mg/dl)
1A	MCO	0	517.1
1A	MCO	3	544.92
1A	MCO	7	-
1A	MCO	10	-
1A	MCO	14	
1A	MCO	17	-
1A	MCO	21	473.25
1A	MCO	24	440.87
1A	MCO	28	434.97
1A	SBO	0	434.97
1A	SBO	3	418.07
1A	SBO	7	392.79
1A	SBO	10	382.87
1A	SBO	14	366.36
1A	SBO	17	478.43
1A	SBO	21	390.55
1A	SBO	24	398.25
1A	SBO	28	464.12
1A	CO	0	464.12
1A	CO	3	506.25
1A	CO	7	388.41
1A	CO	10	481.99
1A	CO	14	-
1A	CO	17	440.83
1A	CO	21	408
1A	CO	24	398.33
1A	CO	28	428.99
1A`	CO	28	-
1B	SBO	0	533.84
1B	SBO	3	502.43
1B	SBO	7	442.53
1B	SBO	10	524.13
1B	SBO	14	439.93
1B	SBO	17	-
1B	SBO	21	572.62
1B	SBO	24	505.29
1B	SBO	28	423.63
1B	CO	0	423.63
1B	CO	3	432.77
1B	CO	7	461.31
1B	CO	10	435.5
1B	CO	14	463.44
1B	CO	17	427.87
1B	CO	21	442.68
1B	CO	24	373.91
1B	CO	28	430.91
1B	MCO	0	430.91

APPENDIX 12 (Continued)

Horse	Diet	Day	Fibrinogen(mg/dl)
1B	MCO	3	395.88
1B	MCO	7	401.86
1B	MCO	10	417.24
1B	MCO	17	450.1
1B	MCO	21	373.2
1B	MCO	24	426.25
1B	MCO	28	408.49
1C	CO	0	526.63
1C	CO	3	494.36
1C	CO	7	473.48
1C	CO	10	513.51
1C	CO	14	519.33
1C	CO	17	464.81
1C	CO	21	442.61
1C	CO	24	446.73
1C	CO	28	454.14
1C	MCO	0	454.14
1C	MCO	3	468.9
1C	MCO	7	435.54
1C	MCO	10	460.71
1C	MCO	14	-
1C	MCO	17	484.43
1C	MCO	21	442.61
1C	MCO	24	446.73
1C	MCO	28	454.14
1C	SBO	0	507.39
1C	SBO	3	408.84
1C	SBO	7	376.55
1C	SBO	10	424.9
1C	SBO	14	377.05
1C	SBO	17	435.99
1C	SBO	21	415.94
1C	SBO	24	420.08
1C	SBO	28	463.24
2A	MCO	0	420.28
2A	MCO	3	428.39
2A	MCO	7	400.77
2A	MCO	10	415.46
2A	MCO	14	427.35
2A	MCO	21	374.25
2A	MCO	24	365.05
2A	MCO	28	397.39
2A	SBO	0	397.39
2A	SBO	3	431.58
2A	SBO	7	369.98
2A	SBO	10	388.21
2A	SBO	14	338.97
2A	SBO	17	366.15

APPENDIX 12 (Continued)

Horse	Diet	Day	Fibrinogen (mg/dl)
2A	SBO	21	323.65
2A	SBO	24	342.58
2A	SBO	28	356.38
2A	CO	0	356.38
2A	CO	3	351.35
2A	CO	7	303.56
2A	CO	10	382.08
2A	CO	17	315.27
2A	CO	21	325.61
2A	CO	24	368.54
2A	CO	28	336.55
2B	SBO	0	547.61
2B	SBO	3	538.83
2B	SBO	7	544.63
2B	SBO	10	513.61
2B	SBO	14	436.56
2B	SBO	21	480.56
2B	SBO	24	472.76
2B	SBO	28	482.71
2B	CO	0	482.71
2B	CO	3	495.81
2B	CO	7	514.2
2B	CO	10	556.91
2B	CO	14	485.4
2B	CO	17	477.77
2B	CO	21	524.46
2B	CO	24	425.61
2B	CO	28	415.48
2B	MCO	0	415.48
2B	MCO	3	409.28
2B	MCO	7	453.45
2B	MCO	10	447.14
2B	MCO	14	-
2B	MCO	17	460.73
2B	MCO	21	418.12
2B	MCO	24	462.55
2B	MCO	28	436.85
2C	CO	0	432.15
2C	CO	3	432.58
2C	CO	7	436.56
2C	CO	10	465.37
2C	CO	14	412.34
2C	CO	17	-
2C	CO	21	422.31
2C	CO	24	457.95
2C	CO	28	444.91
2C	MCO	0	444.91
2C	MCO	3	416.96

APPENDIX 12 (Continued)

Horse	Diet	Day	Fibrinogen (mg/dl)
2C	MCO	7	349.35
2C	MCO	10	373.26
2C	MCO	14	380.61
2C	MCO	17	460.81
2C	MCO	21	398.75
2C	MCO	28	398.09
2C	SBO	0	398.09
2C	SBO	3	444.72
2C	SBO	7	372.06
2C	SBO	10	392.86
2C	SBO	14	-
2C	SBO	17	390.12
2C	SBO	21	342.75
2C	SBO	24	362.11
2C	SBO	28	374.97
3A	MCO	0	449.92
3A	MCO	3	420.78
3A	MCO	7	442.99
3A	MCO	10	490.15
3A	MCO	14	455.95
3A	MCO	17	-
3A	MCO	21	492.59
3A	MCO	24	487.87
3A	MCO	28	389.31
3A	SBO	0	389.31
3A	SBO	3	364.02
3A	SBO	7	401.32
3A	SBO	10	427.75
3A	SBO	14	-
3A	SBO	17	-
3A	SBO	21	-
3A	SBO	24	335.74
3A	SBO	28	372.69
3A	CO	0	372.69
3A	CO	3	333.23
3A	CO	7	353.91
3A	CO	10	-
3A	CO	14	-
3A	CO	17	382.46
3A	CO	21	-
3A	CO	24	-
3A	CO	28	-
3B	SBO	0	529.81
3B	SBO	3	505.84
3B	SBO	7	544.63
3B	SBO	10	490.97
3B	SBO	14	443.05
3B	SBO	21	423.71

APPENDIX 12 (Continued)

Horse	Diet	Day	Fibrinogen (mg/dl)
3B	SBO	24	466.88
3B	SBO	28	435.17
3B	CO	0	435.17
3B	CO	3	322.1
3B	CO	7	453.8
3B	CO	10	412.55
3B	CO	14	405.99
3B	CO	17	432.99
3B	CO	21	423.3
3B	CO	24	385.6
3B	CO	28	355.22
3B	MCO	0	355.22
3B	MCO	3	390.99
3B	MCO	7	431.74
3B	MCO	10	400.36
3B	MCO	17	443.76
3B	MCO	21	450.96
3B	MCO	24	489.3
3B	MCO	28	472.03
3C	CO	0	450.35
3C	CO	3	404.2
3C	CO	7	408.66
3C	CO	10	454.86
3C	CO	14	429.1
3C	CO	17	-
3C	CO	21	427.18
3C	CO	24	423.03
3C	CO	28	347.1
3C	MCO	0	347.1
3C	MCO	3	391.34
3C	MCO	7	377.15
3C	MCO	10	381.29
3C	MCO	14	346.53
3C	MCO	17	375.78
3C	MCO	21	418.54
3C	MCO	24	411.35
3C	MCO	28	394.85
3C	SBO	0	394.85
3C	SBO	3	349.55
3C	SBO	7	353.15
3C	SBO	10	339.19
3C	SBO	14	-
3C	SBO	17	379.05
3C	SBO	21	357.82
3C	SBO	24	371.98
3C	SBO	28	286.58

APPENDIX 13. MEAN PGE2 CONCENTRATION

Horse	Diet	Day	PGE2 (pg/ml)
1A	CO	0	820.1
1A	CO	3	411.7785
1A	CO	10	245.883
1A	CO	17	665.5075
1A	CO	24	2058.685
1A	MCO	0	522.7371
1A	MCO	3	545.9
1A	MCO	10	-
1A	MCO	17	425.68
1A	MCO	24	874.282
1A	SBO	0	630.7964
1A	SBO	3	-
1A	SBO	10	587.2
1A	SBO	17	1145.9
1A	SBO	24	1089.5
1B	MCO	0	1211.58
1B	MCO	3	870.154
1B	MCO	10	-
1B	MCO	17	-
1B	MCO	24	-
1B	SBO	0	906.6753
1B	SBO	3	497.25
1B	SBO	10	576.8
1B	SBO	17	516.15
1B	SBO	24	401.03
1B	CO	0	721.7807
1B	CO	3	1041.045
1B	CO	10	1072.67
1B	CO	17	591.9884
1B	CO	24	556.0778
1C	SBO	0	3119.11
1C	SBO	3	2021.483
1C	SBO	10	2462.471
1C	SBO	17	1644.165
1C	SBO	24	1811.803
1C	CO	0	1893.737
1C	CO	3	1709.2
1C	CO	10	1718.18
1C	CO	17	1424.975
1C	CO	24	1486.725
1C	MCO	0	915.2247
1C	MCO	3	1524.865
1C	MCO	10	1764.549
1C	MCO	17	1098.2
1C	MCO	24	1414.5
2A	CO	0	703.6
2A	CO	3	652.75

APPENDIX 13 (Continued)

Horse	Diet	Day	PGE_2 (pg/ml)
2A	CO	10	477.65
2A	CO	17	639.02
2A	CO	24	643.53
2A	MCO	0	676.8739
2A	MCO	3	501.7
2A	MCO	10	446.75
2A	MCO	17	482.7
2A	MCO	24	524.8
2A	SBO	0	486.9198
2A	SBO	3	400.6
2A	SBO	10	900.025
2A	SBO	17	542.923
2A	SBO	24	522.105
2B	MCO	0	600.355
2B	MCO	3	1354.543
2B	MCO	10	1088.59
2B	MCO	17	1259.215
2B	MCO	24	1028.934
2B	SBO	0	842.3466
2B	SBO	3	1048.76
2B	SBO	10	549.53
2B	SBO	17	818.7795
2B	SBO	24	1263.415
2B	CO	0	627.0946
2B	CO	3	499.6
2B	CO	10	896.504
2B	CO	17	461.2
2B	CO	24	570
2C	SBO	0	2540.42
2C	SBO	10	454.4
2C	SBO	17	2639.45
2C	SBO	24	965.2
2C	CO	0	2100.586
2C	CO	3	680.3
2C	CO	10	1374.305
2C	CO	17	1556.23
2C	CO	24	672.5
2C	MCO	0	1186.399
2C	MCO	3	1021.2
2C	MCO	10	573.9
2C	MCO	17	692.6
2C	MCO	24	-
3A	CO	0	463.148
3A	CO	3	422.15
3A	CO	10	-
3A	CO	17	390.7676
3A	CO	24	398.75
3A	MCO	0	446.9888

APPENDIX 13 (Continued)

Horse	Diet	Day	PGE_2 (pg/ml)
3A	MCO	3	352.1
3A	MCO	10	385.35
3A	MCO	17	446.2
3A	MCO	24	409.2
3A	SBO	0	541.8162
3A	SBO	3	428.8
3A	SBO	10	557.575
3A	SBO	24	380.5
3B	MCO	0	1985.766
3B	MCO	3	1591.57
3B	MCO	10	1937.04
3B	MCO	17	784.4
3B	MCO	24	1818.994
3B	SBO	0	721.983
3B	SBO	3	1487.94
3B	SBO	10	1166.691
3B	SBO	17	1013.903
3B	SBO	24	1661.463
3B	CO	0	816.3435
3B	CO	3	583.4707
3B	CO	10	635.95
3B	CO	17	1362.133
3B	CO	24	447.95
3C	SBO	0	801.5
3C	SBO	3	602.5
3C	SBO	10	624.5
3C	SBO	17	650.1
3C	SBO	24	-
3C	CO	0	1110.612
3C	CO	3	747.1
3C	CO	10	319.4525
3C	CO	17	920.045
3C	CO	24	937.1
3C	MCO	0	807.404
3C	MCO	3	475.322
3C	MCO	10	410.6806
3C	MCO	17	583.2
3C	MCO	24	814.8

APPENDIX 14. ANOVA FOR MEAN FIBRINOGEN CONCENTRATION (mg/dl)

Source	Df	Partial SS	MS	F-value	P-value
Model	8	230396.8	28799.6	14.73	0.000
Period	2	136551.9	68275.96	34.91	0.000
Diet	2	469.65	234.83	0.12	0.886
Diet x Period	4	93283.76	23320.94	11.92	0.000
Residual	206	402894.4	1955.79		
Total	214	438150	2037.9		

APPENDIX 15. ANOVA FOR NORMALIZED MEAN FIBRINOGEN CONCENTRATION (mg/dl)

Source	Df	Partial SS	MS	F-value	P-value
Model	28	137289.3	4903.18	1.9	0.0066
Period	2	4115.88	2057.94	0.8	1.4522
Day	8	47079.16	5884.89	2.28	0.0238
Diet	2	50942.22	25471.11	9.86	0.0001
Diet x Day	16	32889.22	2055.57	0.8	0.689
Residual	186	480299.5	2582.25		
Total	214	438150	2037.9		

APPENDIX 16. ANOVA FOR MEAN PGE2 CONCENTRATION (pg/ml)

Source	Df	Partial SS	MS	F-value	P-value
Model	16	4687082	292942.7	0.92	0.544
Period	2	2898955	1449478	4.57	0.012
Diet	2	485722.5	242861.2	0.77	0.467
Day	4	492146.5	123036.6	0.39	0.816
Diet x Day	8	652812.5	81601.56	0.26	0.977
Residual	108	34243454	317069		
Total	124	38930536	313955.9		

APPENDIX 17.ANOVA FOR NORMALIZED MEAN PGE2 CONCENTRATION (pg/ml)

Source	Df	Partial SS	MS	F-value	P-value
Model	16	5730186	358136.6	0.97	0.499
Period	2	2337642	1168821	3.15	0.046
Diet	2	646834.5	323417.3	0.87	0.421
Day	4	895342.5	223835.6	0.6	0.661
Diet x Day	8	1873666	234208.3	0.63	0.749
Residual	110	40801478	370922.5		
Total	124	46531664	369298.9		

APPENDIX 18. ANOVA FOR MEAN ICTP CONCENTRATION (ng/ml)

Source	Df	Partial SS	MS	F-value	P-value
Model	10	445.69	44.56	1	0.459
Period	2	202.31	101.15	2.26	0.1147
Diet	2	66.49	33.24	0.74	0.481
Day	2	10.43	5.21	0.12	0.89
Diet x Day	4	105.13	26.28	0.59	0.673
Residual	52	2329.16	44.79		
Total	62	2774.86	44.75		

APPENDIX 19. ANOVA FOR NORMALIZED MEAN ICTP CONCENTRATION (ng/ml)

Source	Df	Partial SS	MS	F-value	P-value
Model	8	205.86	25.73	0.5	0.848
Diet	2	11.63	5.82	0.11	0.893
Day	2	84.85	42.43	0.83	0.442
Diet x Day	4	96.07	24.02	0.47	0.757
Residual	54	2760.16	51.11		
Total	62	2966.03	47.84		

APPENDIX 20. ANOVA FOR MEAN ICTP CONCENTRATION (ng/ml) DAY 28

Source	Df	Partial SS	MS	F-value	P-value
Model	2	71.84	35.92	0.62	0.545
Diet	2	71.84	35.92	0.62	0.545
Residual	24	1383.17	57.63		
Total	26	1455.02	55.96		

APPENDIX 21. ANOVA FOR NORMALIZED MEAN ICTP CONCENTRATION (ng/ml) DAY 28

Source	Df	Partial SS	MS	F-value	P-value
Model	2	71.84	35.92	0.59	0.562
Diet	2	71.84	35.92	0.59	0.562
Residual	24	1464.06	61		
Total	26	1535.91	59.07		

APPENDIX 22. ANOVA FOR MEAN OSTEOCALCIN CONCENTRATION (ng/ml)

Source	Df	Partial SS	MS	F-value	P-value
Model	8	1117.14	139.64	0.49	0.861
Diet	2	232.32	116.16	0.41	0.668
Day	2	690.76	345.38	1.21	0.305
Diet x Day	4	158.74	39.69	0.14	0.967
Residual	72	20578.59	285.81		
Total	80	21695.73	271.19		

APPENDIX 23.ANOVA FOR NORMALIZED MEAN OSTEOCALCIN CONCENTRATION (ng/ml)

Source	Df	Partial SS	MS	F-value	P-value
Model	10	2209.87	220.99	1.45	0.177
Period	2	1069.09	534.55	3.51	0.035
Diet	2	274.97	137.49	0.9	0.41
Day	2	714.81	357.41	2.35	0.103
Diet x Day	4	144.5	36.13	0.24	0.916
Residual	70	10667.75	152.39		
Total	80	12877.62	160.97		

APPENDIX 24. ANOVA FOR MEAN MYSTRIC ACID DAY 28

Source	Df	Partial SS	MS	F-value	P-value
Model	8	0.116	0.014	2.54	0.047
Period	2	0.016	0.008	1.46	0.259
Diet	2	0.043	0.021	3.81	0.042
Diet x Period	4	0.056	0.014	2.45	0.083
Residual	18	0.103	0.005		
Total	26	0.219	0.008		

APPENDIX 25. ANOVA FOR MEAN PALMITIC ACID DAY 28

Source	Df	Partial SS	MS	F-value	P-value
Model	8	62.59	7.82	2.83	0.032
Period	2	39.99	19.99	7.23	0.005
Diet	2	16.41	8.2	2.97	0.077
Diet x Period	4	6.18	1.54	0.56	0.694
Residual	18	49.76	2.76		
Total	26				

APPENDIX 26. ANOVA FOR MEAN PALMOLEIC ACID DAY 28

Source	Df	Partial SS	MS	F-value	P-value
Model	8	0.091	0.011	3.64	0.011
Period	2	0.047	0.023	7.64	0.004
Diet	2	0.028	0.014	4.55	0.025
Diet x Period	4	0.014	0.003	1.18	0.351
Residual	18	0.056	0.003		
Total	26	0.147	0.005		

APPENDIX 27. ANOVA FOR MEAN STERIC ACID DAY 28

Source	Df	Partial SS	MS	F-value	P-value
Model	8	107.84	13.48	3.73	0.009
Period	2	61.61	30.81	8.53	0.003
Diet	2	29.44	14.72	4.08	0.035
Diet x Period	4	16.79	4.198	1.16	0.36
Residual	18	65.01	3.61		
Total	26	172.86	6.65		

APPENDIX 28.ANOVA FOR MEAN OLEIC ACID DAY 28

Source	Df	Partial SS	MS	F-value	P-value
Model	8	23.23	2.9	1.45	0.242
Period	2	0.95	0.47	0.24	0.791
Diet	2	1.38	0.69	0.35	0.712
Diet x Period	4	20.89	5.22	2.61	0.07
Residual	18	35.99	1.99		
Total	26	59.23	2.28		

APPENDIX 29. ANOVA FOR MEAN LINOLEIC ACID DAY 28

Source	Df	Partial SS	MS	F-value	P-value
Model	8	54.28	6.78	0.83	0.586
Period	2	11.96	5.98	0.73	0.494
Diet	2	28.83	14.41	1.77	0.198
Diet x Period	4	13.49	3.37	0.41	0.796
Residual	18	146.62	8.15		
Total	26	200.91	7.73		

APPENDIX 30. ANOVA FOR MEAN ALPHA LINOLENIC ACID DAY 28

Source	Df	Partial SS	MS	F-value	P-value
Model	8	0.313	0.039	0.89	0.545
Period	2	0.045	0.022	0.51	0.609
Diet	2	0.117	0.058	1.33	0.289
Diet x Period	4	0.151	0.037	0.86	0.509
Residual	18	0.794	0.044		
Total	26	1.106	0.0426		

APPENDIX 31. ANOVA FOR MEAN ARACHIDONIC ACID DAY 28

Source	Df	Partial SS	MS	F-value	P-value
Model	8	2.09	0.262	8.42	0.0001
Period	2	0.08	0.04	1.29	0.301
Diet	2	1.67	0.836	26.86	0
Diet x Period	4	0.35	0.09	2.77	0.059
Residual	18	0.56	0.03		
Total	26	2.65	0.1		

APPENDIX 32. ANOVA FOR MEAN EICOSAPENTEONIC ACID DAY 28

Source	Df	Partial SS	MS	F-value	P-value
Model	8	11.31	1.41	10.63	0
Period	2	0.32	0.16	1.2	0.323
Diet	2	10.38	5.19	39.01	0
Diet x Period	4	0.61	0.15	1.15	0.365
Residual	18	2.39	0.13		
Total	26	13.71	0.53		

APPENDIX 33. ANOVA FOR MEAN DOCOSAHEXENONIC ACID DAY 28

Source	Df	Partial SS	MS	F-value	P-value
Model	8	13.74	1.71	11.91	0
Period	2	0.1	0.05	0.35	0.708
Diet	2	13.55	6.77	46.96	0
Diet x Period	4	0.09	0.02	0.16	0.956
Residual	18	2.59	0.144		
Total	26	16.34	0.63		

APPENDIX 34. ANOVA FOR % Δ MYSTRIC ACID

Source	Df	Partial SS	MS	F-value	P-value
Model	8	139621.9	17452.74	2.04	0.0995
Period	2	22313.3	11156.65	1.3	0.2957
Diet	2	52572.44	26286.22	3.07	0.071
Diet x Period	4	64736.15	16184.04	1.89	0.1555
Residual	18	153911.4	8550.63		
Total	26	293533.4	11289.74		

APPENDIX 35. ANOVA FOR % Δ PALMITIC ACID

Source	Df	Partial SS	MS	F-value	P-value
Model	8	7199.52	899.94	1.38	0.2687
Period	2	3812.6	1906.3	2.93	0.0792
Diet	2	2178.2	1089.1	1.67	0.2154
Diet x Period	4	1208.72	302.17	0.46	0.761
Residual	18	11712.11	650.67		
Total	26				

APPENDIX 36. ANOVA FOR % Δ PALMOLEIC ACID

Source	Df	Partial SS	MS	F-value	P-value
Model	8	13465.64	1683.21	4.46	0.004
Period	2	6392.26	3196.13	8.48	0.0025
Diet	2	3652.92	1826.45	4.84	0.0208
Diet x Period	4	3420.46	855.12	2.27	0.102
Residual	18	6787.98	377.11		
Total	26	20253.63	778.98		

APPENDIX 37. ANOVA FOR % Δ STERIC ACID

Source	Df	Partial SS	MS	F-value	P-value
Model	8	3284.95	410.62	2.28	0.0702
Period	2	1840.75	920.38	5.1	0.0175
Diet	2	914.07	457.04	2.53	0.1072
Diet x Period	4	530.12	132.53	0.73	0.5801
Residual	18	3246.03	180.33		
Total	26	6530.97	251.19		

APPENDIX 38. ANOVA FOR % Δ OLEIC ACID

Source	Df	Partial SS	MS	F-value	P-value
Model	8	3965.05	495.63	1.04	0.4462
Period	2	128.81	64.41	0.13	0.8749
Diet	2	314.12	157.06	0.33	0.7244
Diet x Period	4	3522.12	880.53	1.84	0.1651
Residual	18	8613.64	478.53		
Total	26	12578.68	483.79		

APPENDIX 39. ANOVA FOR % Δ LINOLEIC ACID

Source	Df	Partial SS	MS	F-value	P-value
Model	8	199.29	24.91	0.78	0.6294
Period	2	43.49	21.74	0.68	0.5208
Diet	2	102.88	51.44	1.6	0.2292
Diet x Period	4	52.91	13.23	0.41	0.798
Residual	18	578.49	32.13		
Total	26	777.79	29.92		

APPENDIX 40. ANOVA FOR % Δ ALPHA LINOLENIC ACID

Source	Df	Partial SS	MS	F-value	P-value
Model	8	82949.68	10368.71	0.94	0.5071
Period	2	7325.68	3662.85	0.33	0.7211
Diet	2	9530.89	4765.45	0.43	0.6549
Diet x Period	4	66093.09	16523.27	1.5	0.2433
Residual	18	197960.9	10997.83		
Total	26	280910.6	10804.25		

APPENDIX 41. ANOVA FOR % Δ ARACHIDONIC ACID

Source	Df	Partial SS	MS	F-value	P-value
Model	8	7642.42	955.3	2.74	0.0361
Period	2	76.18	38.09	0.11	0.8971
Diet	2	5617.64	2808.82	8.06	0.0032
Diet x Period	4	1948.59	487.15	1.4	0.2747
Residual	18	6274.79	348.59		
Total	26	13917.22	535.27		

APPENDIX 42. ANOVA FOR % Δ EICOSAPENTEONIC ACID

Source	Df	Partial SS	MS	F-value	P-value
Model	8	1579858	197482.3	2.44	0.0553
Period	2	214199.7	107099.8	1.32	0.2909
Diet	2	665505	332752.5	4.11	0.0338
Diet x Period	4	700153.6	175038.4	2.16	0.1146
Residual	18	1456536	80918.65		
Total	26	3036394	116784.4		

APPENDIX 43. ANOVA FOR % Δ DOCOSAHEXENONIC ACID

Source	Df	Partial SS	MS	F-value	P-value
Model	8	3488345	436043.2	8.16	0.0001
Period	2	186473.1	93236.56	1.75	0.2028
Diet	2	2832606	1416303	26.51	0.0000
Diet x Period	4	469266.7	117316.7	2.2	0.1105
Residual	18	961476.5	53415.35		
Total	26	4449822	171146.9		

APPENDIX 44. ANOVA FOR MEAN MYSTRIC ACID DAY 0

Source	Df	Partial SS	MS	F-value	P-value
Model	2	0.010381	0.005191	2.75	0.1424
Group	2	0.010381	0.005191	2.75	0.1424
Residual	6	0.011345	0.001891		
Total	8	0.021726	0.002716		

APPENDIX 45. ANOVA FOR MEAN PALMITIC ACID DAY 0

Source	Df	Partial SS	MS	F-value	P-value
Model	2	3.006495	1.503247	0.38	0.7004
Group	2	3.006495	1.503247	0.38	0.7004
Residual	6	23.85022	3.975038		
Total	8	26.85672	3.35709		

APPENDIX 46. ANOVA FOR MEAN PALMOLEIC ACID DAY 0

Source	Df	Partial SS	MS	F-value	P-value
Model	2	0.004352	0.002176	1.15	0.3777
Group	2	0.004352	0.002176	1.15	0.3777
Residual	6	0.011351	0.001892		
Total	8	0.015703	0.001963		

APPENDIX 47. ANOVA FOR MEAN STERIC ACID DAY 0

Source	Df	Partial SS	MS	F-value	P-value
Model	2	2.714702	1.357351	1.31	0.3366
Group	2	2.714702	1.357351	1.31	0.3366
Residual	6	6.203082	1.033847		
Total	8	8.917784	1.114472		

APPENDIX 48. ANOVA FOR MEAN OLEIC ACID DAY 0

Source	Df	Partial SS	MS	F-value	P-value
Model	2	3.105915	1.552950	0.85	0.4732
Group	2	3.105915	1.552950	0.85	0.4732
Residual	6	10.963910	1.827310		
Total	8	14.069830	1.758720		

APPENDIX 49. ANOVA FOR MEAN LINOLEIC ACID DAY 0

Source	Df	Partial SS	MS	F-value	P-value
Model	2	2.25091	1.125459	0.56	0.5985
Group	2	2.25091	1.125459	0.56	0.5985
Residual	6	12.062	2.010334		
Total	8	14.31292	1.789116		

APPENDIX 50. ANOVA FOR MEAN ALPHA LINOLENIC ACID DAY 0

Source	Df	Partial SS	MS	F-value	P-value
Model	2	7.36439	3.68219	10.54	0.0109
Group	2	7.36439	3.68219	10.54	0.0109
Residual	6	2.09537	0.34922		
Total	8	9.45976			

APPENDIX 51. ANOVA FOR MEAN ARACHIDONIC ACID DAY 0

Source	Df	Partial SS	MS	F-value	P-value
Model	2	0.166517	0.083258	4.89	0.055
Group	2	0.166517	0.083258	4.89	0.055
Residual	6	0.102198	0.017033		
Total	8	0.268716	0.033549		

APPENDIX 52. ANOVA FOR MEAN EICOSAPENTEONIC ACID DAY 0

Source	Df	Partial SS	MS	F-value	P-value
Model	2	0.00558	0.00279	3.92	0.0814
Group	2	0.00558	0.00279	3.92	0.0814
Residual	6	0.00427	0.00071		
Total	8	0.00985	0.00123		

APPENDIX 53. ANOVA FOR MEAN DOCOSAHEXENONIC ACID DAY 0

Source	Df	Partial SS	MS	F-value	P-value
Model	2	0.03651	0.01825	4.26	0.0705
Group	2	0.03651	0.01825	4.26	0.0705
Residual	6	0.02569	0.00428		
Total	8	0.06221	0.00777		

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